

**The Mutant-Prevention
Concentration Concept
and its Application to
*Staphylococcus aureus***

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for the Degree of Masters of Science in the
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ABSTRACT

Staphylococcus aureus is a ubiquitous organism causing world-wide morbidity and mortality. This species readily develops resistance to antimicrobial agents. Current dosing strategies are based, in part, on minimum inhibitory concentrations (MICs). This susceptibility test fails to detect the presence of first-step resistant mutants often present in large heterogeneous populations of infecting bacteria. Dosing strategies based on MIC results may, in fact, allow for the selective proliferation of resistant subpopulations. The mutant-prevention concentration (MPC) is the drug concentration at which all first-step resistant mutants will be eradicated along with the susceptible cells. Determination of the mutant-selection window (MSW) is possible using MIC and MPC data. When considered together with achievable drug concentrations in human bodily sites, the MSW helps determine which antimicrobials are likely to select for resistance.

MIC and MPC testing on clinical isolates of methicillin-susceptible (MSSA) and -resistant (MRSA) *S. aureus* was performed. Characterization via the polymerase chain reaction, sequencing, and electron microscopy (EM) was done on selected organisms recovered from MPC studies (MPC-recovered). MIC and MPC testing was performed on organisms isolated sequentially from patients with recurring *S. aureus* infections. Pulsed field gel electrophoresis was performed on these sequential isolates.

Based on the MIC and the MPC values, the most potent agents for systemic MSSA and MRSA infections are gemifloxacin and vancomycin, respectively. Re-testing MPC-recovered populations by the MIC showed increased MIC results compared to the parent populations. Macrolide-resistance genes were discovered in *S. aureus* MPC-recovered populations; in contrast, parental isolates lacked these resistance determinants. EM revealed an increase in cell wall thickness of a vancomycin MPC-recovered population compared to its parental population. Moxifloxacin and vancomycin had the lowest and narrowest MSWs for systemic MSSA and MRSA

infections, respectively, compared to the other agents tested. Sequential isolates showed no change in MIC and MPC values.

The data presented provides evidence for the application of the MPC test to *S. aureus* organisms. The MPC data is significant when determining appropriate dosing strategies aimed at preventing resistance.

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TABLE OF CONTENTS

PERMISSION TO USE	i
ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
LIST OF TABLES	xi
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xvi
1.0 INTRODUCTION	1
1.1 <i>Staphylococcus aureus</i>	1
1.1.1 Characteristics	1
1.1.2 Diseases	1
1.1.3 Resistance	2
1.1.3.1 Resistance to β -lactams	2
1.1.3.2 Resistance to Vancomycin	3
1.1.3.3 Resistance to Macrolides	5
1.1.3.4 Resistance to Quinolones	6
1.2 <i>Streptococcus pneumoniae</i>	6
1.3 Antimicrobial Agents	7
1.3.1 Macrolides	7
1.3.1.1 General Overview	7
1.3.1.2 Mechanism of Action	8
1.3.1.3 Mechanism of Resistance	8
1.3.1.4 Side Effects	9
1.3.2 Clindamycin	9
1.3.2.1 General Overview	9
1.3.2.2 Mechanism of Action	9

1.3.2.3	Mechanism of Resistance	9
1.3.2.4	Side Effects	10
1.3.3	Cephalosporins	10
1.3.3.1	General Overview	10
1.3.3.2	Mechanism of Action	10
1.3.3.3	Mechanism of Resistance	11
1.3.3.4	Side Effects	11
1.3.4	Penicillins	11
1.3.4.1	General Overview	11
1.3.4.2	Mechanism of Action	12
1.3.4.3	Mechanism of Resistance	12
1.3.4.4	Side Effects	12
1.3.5	Quinolones	13
1.3.5.1	General Overview	13
1.3.5.2	Mechanism of Action	13
1.3.5.3	Mechanism of Resistance	14
1.3.5.4	Side Effects	14
1.3.6	Glycopeptides	15
1.3.6.1	General Overview	15
1.3.6.2	Mechanism of Action	15
1.3.6.3	Mechanism of Resistance	15
1.3.6.4	Side Effects	16
1.3.7	Aminoglycosides	16
1.3.7.1	General Overview	16
1.3.7.2	Mechanism of Action	17
1.3.7.3	Mechanism of Resistance	17
1.3.7.4	Side Effects	17
1.3.8	Rifampin	17
1.3.8.1	General Overview	17
1.3.8.2	Mechanism of Action	17
1.3.8.3	Mechanism of Resistance	18

	1.3.8.4 Side Effects	18
1.4	Measurements of Antimicrobial Susceptibility/Resistance	18
1.4.1	Emergence of Resistance	18
1.4.2	Minimum Inhibitory Concentration (MIC) Test	19
1.4.2.1	Standardized Susceptibility Test	19
1.4.2.2	Problems Associated with MIC Testing	20
1.4.3	Mutant-Prevention Concentration (MPC)	22
1.4.3.1	Theory	22
1.4.3.2	Application	23
1.4.4	Mutant-Selection Window (MSW)	24
1.4.4.1	Definition/Relationship to MIC and MPC Values	24
1.4.4.2	Clinical Application	24
1.5	Summary	25
1.6	Objectives	26
2.0	MATERIALS AND METHODS	27
2.1	Standard Laboratory Methods	27
2.1.1	Isolate Collection and Identification	27
2.1.2	Storage of the Bacterial Isolates	27
2.1.3	Susceptibility Testing	28
2.1.3.1	Broth Microdilution	28
2.1.3.2	E-Test	29
2.2	Mutant-Prevention Concentration (MPC)	29
2.2.1	Inoculum	29
2.2.2	Antimicrobial Plates	30
2.2.3	Viable Counts	31
2.3	Characterization of Bacterial Isolates	31
2.3.1	Polymerase Chain Reaction (PCR)	31
2.3.1.1	Primer Preparation	31
2.3.1.2	Setting up the PCR Reaction Tubes	33
2.3.1.3	Thermocycler Settings	33
2.3.1.4	Agarose Gel Electrophoresis	33

2.3.2	Electron Microscopy (EM)	35
2.3.2.1	Preparation of Samples	35
2.3.2.2	Saskatoon Health Region Protocol	35
2.3.2.3	EM Analysis	35
2.3.2.3.1	Statistical Analysis	36
2.3.3	Sequencing	37
2.3.3.1	Preparation From a Gel Sample	37
2.3.3.2	Wizard Kit Protocol	37
2.3.3.3	Sequencing Procedure	38
2.3.4	Pulsed Field Gel Electrophoresis (PFGE)	38
2.3.4.1	Preparation of Samples	38
2.3.4.2	Casting Plugs	38
2.3.4.3	Lysis of Cells in LMP Agarose	38
2.3.4.4	Washing LMP Plugs	38
2.3.4.5	Restriction Enzyme Digestion of LMP Plugs	39
2.3.4.6	Casting Agarose Gel	39
2.3.4.7	Staining and Documentation of the Gel	39
2.4	Miscellaneous Experiments	40
2.4.1	Overcrowding	40
2.4.2	Cefinase	40
3.0	RESULTS	41
3.1	<i>S. aureus</i> Results Against Fluoroquinolones	41
3.2	MIC Results of <i>S. aureus</i> Isolates for Several Antimicrobial Agents	46
3.3	MPC Results of <i>S. aureus</i> Isolates for Several Antimicrobial Agents	47
3.4	Comparison of the MIC and MPC Results	49
3.5	Overcrowding Results	53
3.6	Characterization of MPC-recovered Populations	55
3.6.1	Vancomycin MPC-recovered Populations	55
3.6.2	Azithromycin MPC-recovered Populations	59
3.7	Application of the MIC and MPC Results	62
3.7.1	Pharmacokinetic Curves	62

3.7.1.1	Quinolones	66
3.7.1.1.1	Garenoxacin	66
3.7.1.1.2	Gatifloxacin	66
3.7.1.1.3	Gemifloxacin	69
3.7.1.1.4	Levofloxacin	69
3.7.1.1.5	Moxifloxacin	72
3.7.1.2	Glycopeptide	72
3.7.1.2.1	Vancomycin	72
3.7.1.3	β -lactams	75
3.7.1.3.1	Cloxacillin	75
3.7.1.3.2	Cefazolin	75
3.7.1.4	Macrolide	78
3.7.1.4.1	Azithromycin	78
3.8	Retrospective Multiple Sequential <i>S. aureus</i> Study	78
3.8.1	Isolate Characterization	85
4.0	DISCUSSION	88
4.1	Future Considerations	99
5.0	REFERENCES	101
6.0	APPENDIX A	118
6.1	Solutions and Buffers	118
7.0	APPENDIX B	121
7.1	PFGE Conditions	121
8.0	APPENDIX C	122
8.1	Preparation of Antimicrobial Agents	122
9.0	APPENDIX D	124
9.1	MIC Control Ranges for ATCC <i>S. aureus</i> Strain 29213	124
9.2	MIC Control Ranges for ATCC <i>S. pneumoniae</i> Strain 49619	124
10.0	APPENDIX E	125
10.1	Suppliers	125
10.1.1	Media	125
10.1.2	Antimicrobial Agents	125

10.1.3 Reagents, Chemicals, and Enzymes	125
10.1.4 Disposable Labware	127
10.1.5 Equipment	127

LIST OF TABLES

Table 1.1: Resistance rates (%) of <i>S. aureus</i> to methicillin in Canada and the U.S.A.	4
Table 2.1: Primers used in this study.	32
Table 2.2: Thermocycler conditions for the primers used in this study.	34
Table 3.1: The minimum inhibitory concentration (MIC) and mutant-prevention concentration (MPC) of four fluoroquinolones against 122 clinical isolates of methicillin-susceptible <i>S. aureus</i> .	42
Table 3.2: Comparative minimum inhibitory concentration (MIC) and mutant-prevention concentration (MPC) ($\mu\text{g/ml}$) values of four fluoroquinolones against 22 methicillin-resistant strains of <i>S. aureus</i> .	43
Table 3.3: Fluoroquinolone activity for clinical isolates of <i>S. aureus</i> .	44
Table 3.4: Distribution of minimum inhibitory concentration (MIC) values of <i>S. aureus</i> isolates ($\mu\text{g/ml}$).	48
Table 3.5: Distribution of mutant-prevention concentration (MPC) values of <i>S. aureus</i> isolates ($\mu\text{g/ml}$).	50
Table 3.6: Comparison of the minimum inhibitory concentration (MIC) and the mutant-prevention concentration (MPC) data for <i>S. aureus</i> isolates ($\mu\text{g/ml}$).	51
Table 3.7: Minimum inhibitory concentrations (MICs) of recovered <i>S. aureus</i> isolates from mutant-prevention concentration (MPC) drug plates.	54
Table 3.8: Influence of inoculum volume on the mutant-prevention concentration (MPC) for levofloxacin.	56
Table 3.9: <i>S. pneumoniae</i> isolates with elevated mutant-prevention concentration (MPC) ($\mu\text{g/ml}$) levels and the presence or absence of macrolide-resistance determinants.	64

Table 3.10: Antimicrobial history of patients from whom sequential <i>S. aureus</i> isolates were recovered.	82
Table 3.11: Detection of β -lactamase enzyme from <i>S. aureus</i> isolates using the cefinase test.	83
Table 3.12: Mutant-prevention concentration (MPC) data on the sequential <i>S. aureus</i> isolates ($\mu\text{g/ml}$).	84

LIST OF FIGURES

Figure 1.1: Schematic representation of the behavior of large bacterial populations when exposed to antimicrobial agents at the MIC and MPC.	21
Figure 3.1: Polymerase chain reaction (PCR) for <i>vanA</i> , <i>vanB</i> , and <i>vanC</i> genes in <i>S. aureus</i> isolates with elevated MPC values (MPC-recovered).	57
Figure 3.2: Polymerase chain reaction (PCR) for <i>vanC2/C3</i> and <i>vanD</i> genes in <i>S. aureus</i> isolates with elevated MPC values (MPC-recovered).	57
Figure 3.3: Polymerase chain reaction (PCR) for <i>vanE</i> and <i>vanG</i> genes in <i>S. aureus</i> isolates with elevated MPC values (MPC-recovered).	58
Figure 3.4: Electron microscopy (EM) of a susceptible parent population (#41).	60
Figure 3.5: Electron microscopy (EM) of an intermediate-resistant MPC-recovered population (#41m).	60
Figure 3.6: Polymerase chain reaction (PCR) for <i>ermC</i> in MPC-recovered populations from azithromycin drug plates.	61
Figure 3.7: Polymerase chain reaction (PCR) for <i>ermC</i> in parental isolates of MPC-recovered populations of <i>S. aureus</i> that were positive for <i>ermC</i> .	63
Figure 3.8: Polymerase chain reaction (PCR) for <i>mefA</i> and <i>ermB</i> in <i>S. pneumoniae</i> MPC-recovered populations from MPC plates containing erythromycin.	65
Figure 3.9: Polymerase chain reaction (PCR) for <i>mefA</i> and <i>ermB</i> in <i>S. pneumoniae</i> parental isolates of the MPC-recovered populations showing the presence of either <i>mefA</i> or <i>ermB</i> genes.	65
Figure 3.10: Relationship of serum concentration of garenoxacin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-susceptible <i>S. aureus</i> .	67

Figure 3.11: Relationship of serum concentration of garenoxacin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-resistant <i>S. aureus</i> .	67
Figure 3.12: Relationship of serum concentration of gatifloxacin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-susceptible <i>S. aureus</i> .	68
Figure 3.13: Relationship of serum concentration of gatifloxacin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-resistant <i>S. aureus</i> .	68
Figure 3.14: Relationship of serum concentration of gemifloxacin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-susceptible <i>S. aureus</i> .	70
Figure 3.15: Relationship of serum concentration of gemifloxacin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-resistant <i>S. aureus</i> .	70
Figure 3.16: Relationship of serum concentration of levofloxacin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-susceptible <i>S. aureus</i> .	71
Figure 3.17: Relationship of serum concentration of levofloxacin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-resistant <i>S. aureus</i> .	71
Figure 3.18: Relationship of serum concentration of moxifloxacin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-susceptible <i>S. aureus</i> .	73
Figure 3.19: Relationship of serum concentration of moxifloxacin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-resistant <i>S. aureus</i> .	73
Figure 3.20: Relationship of serum concentration of vancomycin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-susceptible <i>S. aureus</i> .	74

Figure 3.21: Relationship of serum concentration of vancomycin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-resistant <i>S. aureus</i> .	74
Figure 3.22: Relationship of serum concentration of cloxacillin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-susceptible <i>S. aureus</i> .	76
Figure 3.23: Relationship of serum concentration of cloxacillin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-resistant <i>S. aureus</i> .	76
Figure 3.24: Relationship of serum concentration of cefazolin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-susceptible <i>S. aureus</i> .	77
Figure 3.25: Relationship of serum concentration of cefazolin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-resistant <i>S. aureus</i> .	77
Figure 3.26: Relationship of serum concentration of azithromycin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-susceptible <i>S. aureus</i> .	79
Figure 3.27 Relationship of serum concentration of azithromycin to the mutant-prevention concentration ($\mu\text{g/ml}$) for methicillin-resistant <i>S. aureus</i> .	79
Figure 3.28: Pulsed field gel electrophoresis (PFGE) on sequential multiple isolates of <i>S. aureus</i> from patients 1 and 2.	81
Figure 3.29: Pulsed field gel electrophoresis (PFGE) on sequential multiple isolates of <i>S. aureus</i> from patients 3-9.	81
Figure 3.30: Polymerase chain reaction (PCR) for <i>mecA</i> in sequential <i>S. aureus</i> isolates with elevated MPC values to cefotaxime (MPC-elevated).	87
Figure 3.31: Polymerase chain reaction (PCR) for <i>mecA</i> in sequential <i>S. aureus</i> isolates with elevated MPC values to cephalexin and piperacillin/tazobactam (MPC-elevated).	87

LIST OF ABBREVIATIONS

ATCC	American Type Culture and Collection
bp	Base Pairs
CDC	Centre for Disease Control and Prevention
cfu	Colony Forming Units
EM	Electron Microscopy
ETBr	Ethidium Bromide
H ₀	Null Hypothesis
H ₁	Alternative Hypothesis
LMP	Low Melting Point
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant <i>S. aureus</i>
MSSA	Methicillin-Susceptible <i>S. aureus</i>
MPC	Mutant-Prevention Concentration
MSW	Mutant-Selection Window
NCCLS	National Committee for Clinical Laboratory Standards
PBP(s)	Penicillin Binding Protein(s)
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RPC	Resistant Prevention Concentration
s	Sample Standard Deviation
T	Time
TBE	Tris-HCl Boric Acid EDTA Buffer
TE	Tris-HCl EDTA Buffer
THB	Todd Hewitt Broth
TSA	Tryptic Soy Agar

VISA	Vancomycin-Intermediate <i>S. aureus</i>
VRSA	Vancomycin-Resistant <i>S. aureus</i>
Z_c	Critical Value
Z_t	Test Value
μ	Mean
α	Significance Level

1.0 INTRODUCTION

1.1 *Staphylococcus aureus*

1.1.1 Characteristics

Staphylococcus aureus organisms possess a number of characteristics, including traits common to all staphylococci as well as traits unique to *S. aureus*. The characteristics unique to *S. aureus* can be used to differentiate this species from other *Staphylococcus* species. A number of identifiable characteristics of staphylococci include a Gram-positive stain, the presence of catalase, coccoid shape, and an appearance in grape-like clusters. Murray *et al* (1998c) describe the enzyme, coagulase, and protein A as unique characteristics of the species, *S. aureus*. Both protein A and coagulase protect *S. aureus* from immune defenses. *S. aureus* commonly exists as normal flora, but may cause disease under certain circumstances.

1.1.2 Diseases

S. aureus is a ubiquitous organism causing a variety of human diseases either by a toxin or by the direct invasion and destruction of tissue. Murray *et al* (1998c) describe some diseases that may be caused by *S. aureus*. These include toxin-mediated diseases (scalded skin syndrome, toxic shock syndrome, and rapid onset food poisoning), cutaneous infections (cellulitis, impetigo, folliculitis, furuncles, carbuncles, and post-surgical wound infections), urinary tract infections, as well as infections of the respiratory tract. Also, patients may develop bacteremia, osteomyelitis, or endocarditis as a result of a *S. aureus* infection. *S. aureus* is an opportunistic pathogen associated with nosocomial infections. Nosocomial infections are those defined as occurring in patients that have been hospitalized for ≥ 48 hr. *S. aureus* is the most virulent of the staphylococci and, hence, can cause human infection easily. *S. aureus* is the most studied *Staphylococcus* species likely because it is commonly associated with human morbidity and mortality.

1.1.3 Resistance

The tendency for *S. aureus* to develop resistance to antimicrobial agents is a major concern. After the development of resistance to methicillin, clinical isolates of *S. aureus* also resistant to other antimicrobials were isolated. For example, resistance to erythromycin, rifampin, streptomycin, and tetracycline have been found amongst *S. aureus* isolates (Skurray *et al*, 1988). Because of the virulence of this organism in both community and hospital acquired infections, the development of resistance is of great concern.

1.1.3.1 Resistance to β -lactams

Shortly after the introduction of penicillin in the early 1940's, *S. aureus* began developing resistance to this antimicrobial agent (Rammelkamp and Maxon, 1942). It was shown by Bondi and Dietz (1945) that the resistance was due to an enzyme, β -lactamase. β -lactamase degrades the antimicrobial agent before it reaches its target. The genes that code for β -lactamase are plasmid-borne and can, therefore, be easily passed on from bacterial cell to cell (Oliveira *et al*, 2002). As a result, novel semi-synthetic penicillins with the ability to withstand the destructive effect of β -lactamase were developed.

Methicillin, nafcillin, oxacillin, and cloxacillin are among the semi-synthetic penicillins resistant to the destruction by β -lactamase (Rolinson and Sutherland, 1973). Reports of *S. aureus* resistant to methicillin (MRSA), however, emerged in the 1960's (Kagan *et al*, 1964; Seligman, 1966). MRSA possess the 2.1 kb *mecA* gene embedded into a block of 'foreign' DNA which makes up the '*mec* element' or 'staphylococcal chromosomal cassette' (SCC*mec*) (Oliveira *et al*, 2002). The *mec* element incorporates itself into the *S. aureus* chromosome at a specific location. The *mecA* gene codes for a penicillin-binding protein (PBP2A), which has a low affinity for β -lactam antibiotics (Oliveira *et al*, 2002). It is the lack of β -lactam agent binding to the cell that allows the resistant organisms to proliferate in the presence of the newer β -lactam antibiotics. MRSA is common and this organism is easily disseminated. Recent data suggest that 30% of inpatient isolates and 15% of outpatient isolates in the United States are resistant to methicillin (National Nosocomial Infections Surveillance or NNIS, 2000). In Canada, however, the percentage of resistant strains has remained low (<6%)

(Diekema *et al*, 2001). These data are summarized in Table 1.1. If resistance rates continue to rise in Canada as they have in the United States, the mortality and morbidity would be expected to increase significantly among infected Canadian patients.

1.1.3.2 Resistance to Vancomycin

Despite the ability of *S. aureus* organisms to develop resistance to different agents, the majority of strains are susceptible to the glycopeptide vancomycin. Vancomycin resistance in *S. aureus* isolates can be induced experimentally, but because of the difficulty in inducing this resistance, it was thought that the natural tendency to develop resistance to this agent was unlikely for *S. aureus* (Moellering, 1998). Another factor that increased confidence and reliance on vancomycin is that after 20 years of use, no resistant strains of staphylococci had been reported (Srinivasan *et al*, 2002). Because of these factors, vancomycin quickly became the antibiotic of choice for serious MRSA infections (Hamilton-Miller, 2002).

In 1996, however, the first clinical case of vancomycin-intermediate *S. aureus* (VISA) was isolated from a patient in a Japanese hospital (Hiramatsu *et al*, 1997b). Since then, healthcare professionals have been concerned because vancomycin is an important antimicrobial for *S. aureus* infections. Although rare, some individuals have recently had infections caused by *S. aureus* strains with reduced-susceptibility to vancomycin (Srinivasan *et al*, 2002). Vancomycin-resistant *S. aureus* (VRSA) and VISA are those isolates with minimum inhibitory concentration (MIC) values of >32 µg/ml and 8-16 µg/ml, respectively, according to the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 2001a). Research on the mechanism of resistance is ongoing and necessary to help understand the emergence of *S. aureus* with reduced susceptibility to vancomycin.

Very few clinical *S. aureus* isolates have been reported as harboring the vancomycin-resistance genes common to *Enterococcus* species, however, two articles in 2002 reported the existence of a *vanA* genotype in clinical *S. aureus* isolates (CDC, 2002a,b). In the studies of Mu3 and Mu50, two clinical VRSA strains, the resistance was not due to the transfer of either the *vanA* or *vanB* vancomycin-resistance genes (Hiramatsu, 1998). The development of vancomycin-resistant populations may occur as a result of selection for these particular phenotypes from a subpopulation of a

Table 1.1: Resistance rates (%) of *S. aureus* to methicillin in Canada and the U.S.A.

	Resistance Rates (%)	
	U.S.A. ^a	Canada ^b
Inpatient	30	<6
Inpatient Intensive Care Unit (ICU)	50	<6
Out Patient	15	<6

^aNational Nosocomial Infections Surveillance (NNIS), 2000

^bDiekema *et al*, 2001

heterogeneous population of cells (Ike *et al*, 2001). A heterogeneous population of cells is a population consisting of a majority of susceptible cells and far fewer resistant cells that are created by mutation(s) or acquisition of resistance genes (Srinivasan *et al*, 2002). One difference noted between a VISA and vancomycin-susceptible *S. aureus* (VSSA) is the thickening of the cell wall in VISA isolates (Smith *et al.*, 1999b). One theory of resistance based on the thickening of the cell wall comes from Hiramatsu *et al* (2002). The authors state, "...the thickening causes the increased intra-cell-wall trapping of vancomycin molecules and prevents their reaching targets on the cytoplasmic membrane" (Hiramatsu *et al*, 2002). The studies of VRSA are preliminary and though little is known about the mechanism of resistance, it is suggested that thickening of the cell wall may be a phenotypic resistance determinant for *S. aureus* isolates with decreased susceptibility to vancomycin (Cui *et al*, 2003).

Hiramatsu *et al* (2002) state that vancomycin resistance is acquired by a multi-step genetic phenomenon. The authors found that VRSA organisms are extremely unstable and lose their resistant phenotype when there is a lack of vancomycin selective pressure. The resistant strain is then replaced by a faster-growing revertant. They conclude that "...hetero-VRSA is the essence of vancomycin resistance from which vancomycin selects VRSA and to which it returns when selective pressure is lifted" (Hiramatsu *et al*, 2002). If vancomycin resistance were to develop worldwide and spread easily, this would be devastating because of the current reliance on vancomycin for therapy in infected patients.

Healthcare professionals are now focusing on alternative therapies for MRSA infections. Other antimicrobials showing clinical success with MRSA infections include linezolid, quinupristin/dalfopristin, and teicoplanin. Because of the possibility of resistance to both old and new antimicrobials, reliance on one agent should be avoided.

1.1.3.3 Resistance to Macrolides

Recently, the macrolides have become more commonly used for treatment of *S. aureus* infections. These agents are being used extensively in not only human clinical practice, but also in the poultry and livestock industries (Devriese, 1980; Owens *et al*, 1988; Brown and Scassera, 1990; Skeeles, 1991; Westh *et al*, 1991; Nawaz *et al*, 2000).

The widespread plus prolonged use of these agents has led to an increase in resistance to the macrolides (Weisblum and Demohn, 1969; Andreumont *et al*, 1986; Arthur *et al*, 1987; Nawaz *et al*, 2000). Resistance to macrolides is accomplished by either target modification or an efflux system. Target modification is carried out by the erythromycin resistance methylase (*erm*) genes and efflux is a result of the macrolide efflux genes (*mefA/E* gene or the *msrA/B* gene). If resistance to macrolides continues to increase, it will compromise the use of this family of antimicrobial agents.

1.1.3.4 Resistance to Quinolones

The quinolone agents are potent antimicrobials that have proven successful for treating many infections. Unfortunately, many hospital-acquired *S. aureus* isolates demonstrate resistance to quinolones (Blumberg *et al*, 1991; Neu, 1992; Acar and Goldstein, 1997; Zhao *et al*, 2003). Many community-acquired isolates, however, are still susceptible to quinolones (Schito *et al*, 1996; Gorak *et al*, 1999; Lister, 2000; Ma *et al*, 2002; Zhao *et al*, 2003). In a study done by Almer *et al* in 2002, 49% of community-acquired *S. aureus* were susceptible to quinolone agents. In Saskatoon, over 95% of *S. aureus* isolates remain susceptible to ciprofloxacin (Blondeau-personal communication). This creates optimism in the ability of quinolones to successfully treat infections caused by *S. aureus*. As novel quinolones are being developed, the possibility of resistance should be considered and avoided through proper use of these agents as to maintain their effectiveness.

1.2 *Streptococcus pneumoniae*

Although the focus of this project was essentially with the organism *S. aureus*, some data were assessed for *Streptococcus pneumoniae* isolates. *S. pneumoniae* is a Gram-positive organism causing bacteremia, meningitis, otitis media, pneumonia, and sinusitis (Murray *et al*, 1998c). Resistance of this organism to antimicrobial agents is increasing worldwide. Macrolides are being used in patients with respiratory tract infections caused by pneumococci. Pihlajamaki *et al* (2001) have studied the correlation between macrolide use and resistance in different geographical regions. They found that the prevalence of macrolide-resistant *S. pneumoniae* was higher in regions with greater macrolide use. *S. pneumoniae* may become resistant to macrolides by target modification or efflux (Gay and Stephens, 2001). Efflux is accomplished by

the *mefA/E* gene and results in low to moderate levels of resistance (Montanari *et al*, 2003). Target modification is accomplished either by the *erm* class methylases which results in high level resistance or by mutations in the 23S rRNA (Montanari *et al*, 2003). Strains with inducible resistance have *ermB* or *ermB* plus *mefE* (Nishijima *et al*, 1999). Strains with non-inducible resistance have only *mefE* (Nishijima *et al*, 1999). *MefA* and *mefE* are very similar and are often not distinguished by conventional Polymerase Chain Reaction (PCR) methods, therefore, these genes are regarded as a single class often referred to as *mefA* (Sutcliffe *et al*, 1996; Perez-Trallero *et al*, 2001). *S. pneumoniae* strains with intermediate-resistance or resistance to penicillin are more likely to be concomitantly resistant to other classes of antimicrobial agents, including macrolides, than *S. pneumoniae* strains sensitive to penicillin (Noreddin *et al*, 2002). *S. pneumoniae* is a clinically important pathogen and the recent increase in resistance observed in this organism is disconcerting.

1.3 Antimicrobial Agents

1.3.1 Macrolides

1.3.1.1 General Overview

Macrolide agents include erythromycin, josamycin, midecamycin, oleandomycin, rosaramycin, roxithromycin, and spiramycin (Periti *et al*, 1989). Erythromycin, the first macrolide antibiotic, was isolated from *Streptomyces erythreus* and was marketed in 1952 (Blondeau *et al*, 2002a). Macrolides are basic and are mostly absorbed in the alkaline intestinal environment (Periti *et al*, 1989). The basic structure of macrolides is the macrolide ring bound to two sugars (desosamine and cladinose) (Zuckerman, 2000). Azithromycin and clarithromycin are new azalide agents that were semi-synthetically modified from original macrolides (Periti *et al*, 1989). Azithromycin contains a nitrogen atom in the macrolide ring resulting in a 15-membered structure, whereas, clarithromycin is a 14-membered compound which lacks the nitrogen atom in its structure. (Danesi *et al*, 2003). Approval of azithromycin by the Food and Drug Administration (FDA) occurred in 1992. This agent was developed to better withstand acid conditions compared to the previously developed macrolides, such as erythromycin (Zuckerman, 2000). Azithromycin and clarithromycin, the “new” macrolides, have a much lower incidence of side effects as well as a broader spectrum of activity compared

to older macrolides (Blondeau *et al*, 2002a). This family of antibiotics enters pleural and ascitic fluids, middle ear exudates, and the sputum, however, these drugs do not normally enter the cerebral spinal fluid in clinically useful concentrations (Periti *et al*, 1989). The macrolides are often used as an alternative to penicillin (Pihlajamaki *et al*, 2001). These agents are becoming more common in treating upper respiratory infections as well as some *S. aureus* infections. The broad spectrum of these agents allows them to be useful in the treatment of a variety of bacterial infections (Berry *et al*, 1998; Donati *et al*, 1999; Danesi *et al*, 2003).

1.3.1.2 Mechanism of Action

Macrolide antibiotics work by blocking protein synthesis. These agents reversibly bind to the 50S ribosomal subunit (Periti *et al*, 1989). This, in turn, blocks transpeptidation or translocation, hence preventing protein chain elongation. This family of antimicrobials is bacteriostatic which means that they do not kill the cells, but prevent them from growing.

1.3.1.3 Mechanism of Resistance

Resistance toward macrolides may evolve in different ways. Methylation of the 23S ribosomal RNA is one way in which an organism may become resistant to a macrolide (Lim *et al*, 2002). This change prevents the antibiotic from binding to the target site. The antibiotic cannot act on the organism without binding to its target site, therefore, the organism is rendered resistant. This resistance is achieved via one or more of the *erm* genes (Wondrack *et al*, 1996; Pechere, 2001; Lim *et al*, 2002). There are seventeen different types of *erm* genes known (Nawaz *et al*, 2000), however, *ermA*, *ermB*, and *ermC* are most common in staphylococci. Another mechanism of resistance that has been observed is efflux (Lim *et al*, 2002). This is the pumping out of the antibiotic from the cell, preventing it from reaching its target. This mechanism of resistance is accomplished by the macrolide efflux gene (*mefA/E*) in *S. pneumoniae* (Lim *et al*, 2002) or by the macrolide-specific resistance gene (*msrA/msrB*) in *S. aureus* (Matsuoka *et al*, 2003). Resistance to macrolides may also occur via enzymatic degradation (Pechere, 2001). Many different enzymes conferring macrolide resistance have been discovered in *Enterobacteriaceae* and staphylococci (Wondrack *et al*, 1996; Pechere, 2001). This resistance may have evolved naturally to ensure survival of

bacteria in environments harboring the antimicrobial products of *Streptomyces* species (Pechere, 2001).

1.3.1.4 Side Effects

The newer macrolides such as azithromycin and clarithromycin were developed to decrease the severity of the side effects that accompanied the older macrolides. Some severe side effects more common in the older macrolides include cholestatic hepatitis (Hautekeele, 1995) and cranial nerve toxicity (Snavey, 1984). Some less serious, but more frequent side effects in the earlier developed macrolides include nausea, cramping, and diarrhea (Blondeau *et al*, 2002a). The newer macrolides (developed in the 1980's and marketed in the 1990's to present day) are clinically efficacious as well as having fewer, minor side effects.

1.3.2 Clindamycin

1.3.2.1 General Overview

Clindamycin belongs to the family of lincosamides and was isolated from the species *Streptomyces lincolnensis* (Murray *et al*, 1998b). Its use has been appreciated for treating malaria (Pukrittayakamee *et al*, 2004), toxoplasmosis (Fung and Kirschenbaum, 1996), and babesiosis (Krause, 2003). As well, clindamycin is active against staphylococci and streptococci (Leclercq, 2002). Delivery of this agent is accomplished orally, intravenously, or intramuscularly (Gilbert *et al*, 2002).

1.3.2.2 Mechanism of Action

Clindamycin acts on bacteria in a similar manner as the macrolides. Clindamycin binds to the 50S ribosomal subunit, blocking protein elongation. This agent interferes with the binding of the tRNA complex, thereby inhibiting peptidyl transferase, an enzyme responsible for transferring linked amino acids to the aminoacyl site from the peptidyl site in the tRNA (Murray *et al*, 1998b). This agent does not result in the death of the organism, but inhibits the organism from further growth.

1.3.2.3 Mechanism of Resistance

Leclercq (2002) reviews the three mechanisms of resistance to lincosamides. Methylation of the 23S rRNA alters the drug target site. This resistance is plasmid-mediated and confers broad-spectrum resistance to both macrolides and lincosamides. Efflux and drug inactivation are the other two mechanisms of resistance that have been

observed in organisms resistant to lincosamides. These three resistance mechanisms have been found naturally in macrolide and lincosamide producers to protect themselves against the antimicrobial products they produce.

1.3.2.4 Side Effects

Serious side effects that have been connected to the use of clindamycin include hepatitis and pseudomembranous colitis (Gilbert *et al*, 2002). More commonly observed, however, are the less serious adverse effects of fever and rash.

1.3.3 Cephalosporins

1.3.3.1 General Overview

The antibiotics cefazolin, cephalexin, cefuroxime, and cefotaxime belong to the family of cephalosporins. Cephalosporin agents, originally recovered from the mold *Cephalosporium acremonium*, are β -lactam antibiotics comprised of a β -lactam ring fused with a dihydrothiazine ring (Nightingale *et al*, 1975). Cefazolin is a first-generation, semi-synthetic cephalosporin and is administered parenterally (intramuscularly or intravenously) (Nightingale *et al*, 1975; Gilbert *et al*, 2002). Cephalexin, like cefazolin, is a first generation cephalosporin, but unlike cefazolin, cephalexin is administered orally (Gilbert *et al*, 2002). Cefuroxime and Cefotaxime are second and third-generation cephalosporins, respectively. Cefuroxime is administered either orally or intravenously, whereas cefotaxime is administered parenterally. Cefazolin levels remain high in the blood for approximately 90 min (Nishida *et al*, 1969; Shibata and Fujii, 1970; Lopez-Sosa *et al*, 1993). Also, cefazolin is almost completely recovered in the urine after intramuscular or intravenous administration (Nishida *et al*, 1969; Shibata and Fujii, 1970). Cephalosporins initially possessed a broad spectrum of activity against most Gram-positive and Gram-negative bacteria (Nightingale *et al*, 1975). Unfortunately, some Gram-negative organisms have developed resistance to these agents and this has compromised their use in infected patients (Murray *et al*, 1998b).

1.3.3.2 Mechanism of Action

Cephalosporins act on their target cells by interfering with the penicillin-binding proteins (PBPs). This interference results in the destabilization of the cell wall, leading to cell death.

1.3.3.3 Mechanism of Resistance

Resistance to cephalosporins may occur via β -lactamase (described in section 1.1.3.1) (Livermore, 1987). Mutations in *fusA*, the gene that codes for an elongation factor, may also result in resistance to cephalosporins (O'Neill *et al*, 2002). The *mecA* gene may also confer resistance to cephalosporins by altering the penicillin binding site, preventing cephalosporins from binding to the target protein (Mandell, 1998).

1.3.3.4 Side Effects

Gilbert *et al* (2002) list side effects that occur with cephalosporin treatment. Significant hematological and hepatic effects have been correlated with cephalosporin treatment. Frequent side effects include rash, fever, and diarrhea.

1.3.4 Penicillins

1.3.4.1 General Overview

Like the cephalosporin agents, penicillin agents are also β -lactams. Murray *et al* (1998b) describe penicillin in detail. The basic structure is an organic acid with a β -lactam ring fused to a 5-membered thiazolidine ring. Penicillin was first obtained from a mold called *Penicillium chrysogenum* and from this mold natural penicillin agents like benzylpenicillin (penicillin G) and phenoxymethyl penicillin (penicillin V) were developed. Penicillin was one of the first antibiotics on the market and proved to be very effective with low toxicity. Shortly after its introduction, however, resistance emerged to this drug. New β -lactam agents were created to bypass the resistance displayed by *S. aureus*. For example, cloxacillin, dicloxacillin, methicillin, nafcillin, and oxacillin are β -lactamase-resistant. Cloxacillin is absorbed from the gastrointestinal tract and a small amount of the drug is lost due to acid hydrolyzation (Soldin *et al*, 1980). This is a major disadvantage for cloxacillin, however, once absorbed, it is very effective against β -lactamase-producing *S. aureus* strains (Nauta and Mattie, 1975; Soldin *et al*, 1980).

Although their spectrum of activity is limited, the aminopenicillins (ampicillin and amoxicillin) were the first penicillin agents active against Gram-negative bacilli (Murray *et al*, 1998b). Later, the carboxypenicillins (carbenicillin and ticarcillin) and the ureidopenicillins (mezlocillin and piperacillin) were created and have a broad spectrum of activity against Gram-negative bacteria. Piperacillin/tazobactam was

subsequently developed as a combination of piperacillin and the β -lactamase inhibitor, tazobactam. Because tazobactam is structurally similar to β -lactam drugs, it binds to β -lactamase enzymes, preventing them from destroying the piperacillin. Penicillins can be administered orally, intramuscularly, or intravenously.

1.3.4.2 Mechanism of Action

Penicillins act by binding to the PBP situated within the peptidoglycan of the cell, thus causing destabilization of the cell wall (preventing cell wall synthesis) (Tomasz, 1979). The PBPs are the enzymes responsible for the catalysis of reactions necessary for cell wall synthesis and include D-alanyl-D-alanine-carboxy-peptidases and transpeptidases (Murray *et al*, 1998a). Penicillin causes the cell to release autolytic enzymes that degrade the cell wall (Tomasz, 1979). Degradation of the cell wall results in the cell bursting. In addition to cell wall destruction, some of the more recently developed penicillin agents have the ability to inhibit or resist bacterial β -lactamases.

1.3.4.3 Mechanism of Resistance

Organisms may become resistant to penicillin in a number of ways. Resistance may be accomplished through the failure of the antimicrobial agent to cross the membrane and, hence, reach the drug's target. This is primarily associated with Gram-negative organisms. Two other mechanisms of resistance that are common to both Gram-positive and Gram-negative bacteria are the alteration of the PBPs and the production of β -lactamase. Many *S. aureus* isolates have now developed resistance to the newer penicillin agents by acquiring the *mec* element. This gene was discussed in section 1.1.3.1.

1.3.4.4 Side Effects

Like most other antibiotics, with the administration of penicillin, there is a risk of side effects. Possible severe side effects include hypersensitivity, seizures, platelet dysfunction, neutropenia, hemolytic anemia, liver problems, encephalopathy, and interstitial nephritis (RxList.com, 2003a). When one is allergic to penicillin, an alternative form of antimicrobial therapy is necessary. Some commonly experienced and less serious side effects include rash, diarrhea, chills, fever, edema, and electrolyte disturbance (RxList.com, 2003a).

1.3.5 Quinolones

1.3.5.1 General Overview

In 1962, the first quinolone, nalidixic acid, was introduced (Leshner *et al*, 1962). The development of fluoroquinolones involved the addition of fluorine at the six-position of a quinolone agent (Goldstein *et al*, 2002). The classical fluoroquinolones included ciprofloxacin, norfloxacin, and ofloxacin (Naber *et al*, 2001). New fluoroquinolones have been developed because the classical fluoroquinolones have a limited spectrum of activity (Lubasch *et al*, 2000). New fluoroquinolones include gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin. Garenoxacin (still undergoing investigation and not yet licensed anywhere in the world) is a new des-F(6)quinolone that does not possess the characteristic fluorine at the six-position (Goldstein *et al*, 2002). Garenoxacin has been shown to be very potent (Goldstein *et al*, 2002), which is contradictory to the belief that the potency of recently developed fluoroquinolones is a result of the C-6 fluorine (Ryan *et al*, 2002). Gatifloxacin and moxifloxacin have a methoxy group at the C-8 position and this addition results in increased activity against Gram-positive bacteria (Fung-Tome *et al*, 2001). New derivatives with C-8 chlorine and C-8 methoxy moieties exhibit increased activity against quinolone-resistant mutants (Zhao *et al*, 1997; Lu *et al*, 2001). The derivatives of the fluoroquinolones have an extended spectrum of activity and improved pharmacokinetic properties compared to the classical fluoroquinolones (Lubasch *et al*, 2000). Quinolones are useful for treating a wide variety of bacterial infections, including skin and skin structure infections caused by *S. aureus*, and are often considered as an alternative to penicillin (Lubasch *et al*, 2000; Blondeau *et al*, 2001). The use of quinolones for *S. aureus* infections has increased due to widespread resistance to penicillin and increasing resistance to the macrolides (Li and Nikaido, 2004).

1.3.5.2 Mechanism of Action

Quinolone agents exert their bactericidal effects by inhibiting topoisomerase IV or DNA gyrase (topoisomerase II), enzymes critical for DNA replication (Harley *et al*, 1993). The fluoroquinolones prohibit the supercoiling of DNA, thus inhibiting DNA synthesis. Two alpha (GyrA encoded by the *gyrA* gene) and two beta (GyrB encoded

by the *gyrB* gene) subunits make up DNA gyrase and the quinolones bind to the alpha subunits (Murray *et al*, 1998b). Topoisomerase consists of a tetramer and is encoded by *parC* and *parE* genes (termed *glrA* and *glrB*, respectively, in *S. aureus*) (Ferrero *et al*, 1994). DNA supercoiling is imperative for the survival of a cell. Quinolones inhibit supercoiling, therefore, the cells cannot survive in the presence of these agents.

1.3.5.3 Mechanism of Resistance

Quinolone resistance has been established worldwide and fluoroquinolone resistance is appearing rapidly despite the fact that fluoroquinolones are novel antibiotics (Chen *et al*, 1993; Li and Nikaido, 2004). The principal mechanism for resistance to fluoroquinolones is alteration of the antimicrobial target as a result of a mutation in the quinolone resistance-determining regions (QRDR) (Yoshida *et al*, 1990). These mutations are specifically within the genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) (Johnson *et al*, 2003). Resistance to quinolones occurs in a step-wise fashion, increasing the level of resistance when in the presence of differing concentrations of the quinolone agent (Allen, 2003). Efflux has also been observed in organisms that are resistant to quinolones (Poole, 2000). Newer fluoroquinolone agents appear to be more effective against multi-drug resistant bacteria compared to older quinolones.

1.3.5.4 Side Effects

Significant side effects associated with fluoroquinolones are uncommon. Gilbert *et al* (2002) state a number of possible side effects with fluoroquinolones. Depending on the agent, one potential side effect is photophobia. Also, there is a potential for these agents to have adverse effects on joint cartilage. This potential side effect is based on observations in animal studies and, to date, no human clinical cases have demonstrated this effect. However, these agents are not recommended for children <16 yrs of age because of the potential developmental problems in joint cartilage that may be associated with these agents. For the same reason, quinolones are not recommended for use in pregnant women as these drugs have been shown to cross the placenta and may, therefore, have detrimental effects on the development of the fetus (Giamarellou *et al*, 1989). Other side effects frequently associated with these drugs

include gastro-intestinal upset, central nervous system toxicity, and allergic reactions. These side effects are minimal for the new fluoroquinolones.

1.3.6 Glycopeptides

1.3.6.1 General Overview

Glycopeptides are complex heterocyclic molecules that consist of a heptapeptide backbone with various attached sugars (Pfeiffer, 1981). These agents were used in patients that had allergies to penicillin before alternative agents were available. Glycopeptides are delivered principally by the intravenous route. One of the most successful glycopeptides is vancomycin. It was obtained originally from *Streptomyces orientalis* (McCormick *et al*, 1955-1956). Vancomycin is the antibiotic of choice for life-threatening MRSA infections (Hamilton-Miller, 2002). Neither vancomycin, nor any other glycopeptide, is used for Gram-negative bacteria because the large size of the compounds makes it impossible for them to penetrate the outer membrane and reach their target in the peptidoglycan layer (Geisel *et al*, 2001).

1.3.6.2 Mechanism of Action

Glycopeptides prevent the addition of new building blocks to the growing cell wall. These agents interact with the D-alanyl-D-alanine ends of the pentapeptide chains, which interfere with the formation of the bridges between the peptidoglycan chains (Murray *et al*, 1998b). The cell wall of an organism is essential for the survival of the cell. Without the proper addition of the building blocks and maintenance of the cell wall, the organism will perish.

1.3.6.3 Mechanism of Resistance

Vancomycin-resistant enterococci (VRE) are commonly spread throughout a hospital and are difficult to control. There are a number of vancomycin-resistance genes that have been discovered in enterococci. These include *vanA*, *vanB*, *vanC* (Dutka-Malen *et al*, 1995), *vanC2/3* (Kariyama *et al*, 2000), *vanD* (Perichon *et al*, 1997), *vanE* (Fines *et al*, 1999), and *vanG* (McKessar *et al*, 2000). These vancomycin-resistance genes have seriously compromised the usefulness of vancomycin for enterococcal infections. The products of these genes create an alteration in the pentapeptide terminus, prohibiting the glycopeptide from binding to its target (Woodford, 1998). Within the past six years, there have been reports of clinical isolates

of *S. aureus* with intermediate-resistant to resistant phenotypes against vancomycin (Hiramatsu *et al*, 1997a,b; Rotun *et al*, 1999; Smith *et al*, 1999b; CDC, 2002a,b; Sakoulas *et al*, 2002; Fridkin, 2003). Although the mechanism of resistance is unknown, the cell wall of *S. aureus* isolates with intermediate-resistant or resistant phenotypes is thicker than the cell wall of susceptible *S. aureus* or MRSA (Smith *et al*, 1999b). Increasing vancomycin-resistance will decrease the overall efficacy of glycopeptides, therefore, studies should be performed to help understand the resistance mechanisms.

1.3.6.4 Side Effects

Nephrotoxicity and ototoxicity of glycopeptides is a concern, especially when administered with an aminoglycoside (Wenk *et al*, 1984; Gilbert *et al*, 2002). Vancomycin has been associated with an infusion-related reaction called red man syndrome (Sivagnanam and Deleu, 2003). This syndrome typically consists of an erythematous rash involving the face, neck, and upper torso. Glycopeptides have been known to cause vomiting and tachycardia (WholeHealthMD.com). Some frequent side effects experienced with glycopeptides include fever, chills, dizziness, and phlebitis (Gilbert *et al*, 2002).

1.3.7 Aminoglycosides

1.3.7.1 General Overview

A review article written by Tolmasky (2000) describes the structure, isolation, and development of aminoglycoside agents. These agents consist of amino sugars associated with glycosidic bonds and linked to an aminocyclitol ring. Some examples of aminoglycosides are kanamycin, neomycin, streptomycin, tobramycin (all isolated from *Streptomyces*), and gentamicin and sisomicin (isolated from *Micromonospora*). Amikacin and netilmicin were derived synthetically from kanamycin and sisomicin. The most common antimicrobials in this family are gentamicin and tobramycin which both have a broad spectrum of activity. These agents are used to treat serious infections caused by Gram-negative bacteria and, when combined with other agents, to treat some Gram-positive bacteria.

1.3.7.2 Mechanism of Action

Aminoglycosides inhibit protein synthesis by binding irreversibly to the 30S ribosomal proteins. This binding to the 30S ribosomal proteins results in the production of aberrant proteins due to misreading of the mRNA and this attachment also causes premature release of the ribosome from the mRNA, resulting in protein synthesis interruption (Tolmasky, 2000). The bacterial cell requires properly assembled proteins for survival, therefore, the action of aminoglycosides is bactericidal.

1.3.7.3 Mechanism of Resistance

Tolmasky (2000) describes three mechanisms by which an organism may become resistant to an aminoglycoside. A mutation in the ribosome-binding site can prevent the aminoglycoside from attaching to the 30S ribosomal subunit. Another mechanism of resistance is the decreased uptake of the aminoglycoside accomplished by an efflux system. Resistance can also be due to enzymatic modification (the most commonly observed mechanism of resistance to aminoglycosides). Adenylation, acetylation, and phosphorylation of the amino and hydroxyl groups of aminoglycosides occur often in bacteria that are resistant to aminoglycosides.

1.3.7.4 Side Effects

Aminoglycosides are potentially toxic and this toxicity is dependent on the dose. Aminoglycosides may cause nephrotoxicity, ototoxicity, and in rare cases, neuromuscular blockade (Gilbert *et al*, 2002).

1.3.8 Rifampin

1.3.8.1 General Overview

This agent is a member of the ansamycin family of antibiotics. Rifampin was derived from *Streptomyces mediterranei* (Morris *et al*, 1993). Rifampin has shown clinical success against *Mycobacterium tuberculosis* and is also very active against staphylococci and streptococci as well as other clinically significant Gram-positive organisms (Murray *et al*, 1998b).

1.3.8.2 Mechanism of Action

In a review article written by Morris *et al* (1993), the authors describe the mechanism of action of rifampin. Rifampin exerts its effects by binding to the DNA-

dependent RNA polymerase. The result of this binding is the inhibition of RNA synthesis, resulting in cell death.

1.3.8.3 Mechanism of Resistance

Resistance develops quickly to rifampin, so this agent is often administered with another effective antimicrobial agent. Almost all isolates resistant to rifampin have a mutation in the beta subunit of the RNA polymerase (Ramaswamy and Musser, 1998). Gram-negative bacteria are resistant to rifampin intrinsically due to a decreased uptake of the drug (Murray *et al*, 1998b).

1.3.8.4 Side Effects

Common side effects associated with the administration of rifampin include heartburn, flatulence, cramps, diarrhea, fever, headache, drowsiness, fatigue, rash, and dizziness (RxList.com, 2003b). Some serious, but rare side effects include hepatitis, pseudomembranous colitis, thrombocytopenia, transient leukemia, hemolytic anemia, anaphylaxis, and decreased hemoglobin (RxList.com, 2003b).

1.4 Measurements of Antimicrobial Susceptibility/Resistance

1.4.1 Emergence of Resistance

Millions of people suffer from infections caused by antimicrobial resistant bacteria. Often, infections are not entirely cleared by an antibiotic because the bacteria undergo a change that allows them to resist the antimicrobial's effects and continue to proliferate in the presence of the agent. There is great concern about infections caused by resistant bacteria because these types of infections may lead to treatment failure in a patient, increase overall costs, and result in greater morbidity and mortality among patients (Drlica and Schmitz, 2002).

Gould and MacKenzie (2002) describe many ways in which a resistant population may emerge. A minority population of pre-existing resistant cells may be selected out of a heterogeneous population of cells. A resistant population may also proliferate after the selection of induced or random mutations. This type of resistance is called *de novo* resistance and occurs more often as a stress response as well as when the inoculum of the organism is large. Resistance may develop via the introduction of DNA from plasmids, phages, mosaic genes, and transposons which carry resistance

determinants. Bacteria are creative in their ability to develop resistance in a society that relies daily on antimicrobial therapy.

There are many factors that play a role in the emergence of resistant bacteria. Studies have shown that the cessation of therapy coincides with a decrease in resistance (Smith *et al*, 1987). Also, most investigators will agree that increased use of antibiotics correlates with increase of resistant organisms present today (Pihlajamaki *et al*, 2001). “Clearly, the most important factor in the emergence of antimicrobial resistance with *S. pneumoniae*, however, is the selective pressure of antimicrobial agents.” (Doern, 2001). Some believe that it is not only the overuse of antibiotics that causes an increase in resistance, but also the way in which antimicrobials are used that contribute to the emergence of resistance (Drlica and Schmitz, 2002). Investigators believe that it is likely resistant mutants are selected by antimicrobial therapy (Drlica and Schmitz, 2002). Also, the inevitable, unintentional exposure of normal flora from both the environment and the host to antibiotics is a factor in increasing resistance rates (Gould and MacKenzie, 2002). Also increasing the prevalence of resistant organisms is the use of antimicrobial agents in agriculture (Endtz *et al*, 1991; Gaunt and Piddock, 1996; Garau *et al*, 1999; Smith *et al*, 1999a; Zhao and Drlica, 2001). The presence of selective pressure increases the possibility of proliferating resistant bacteria. Another factor that may contribute to the increase in resistant organisms is the fact that many patients’ immune systems are weak as a result of aging, Human Immunodeficiency Virus, radiotherapy, the use of immunosuppressive drugs, etc. (Zhao and Drlica, 2001). The weakened immune system reduces the ability to ward off bacteria, thereby allowing the resistant bacterial populations to proliferate and possibly infect other susceptible patients within a hospital. It is likely that these factors have contributed to the overall increase in resistance observed in bacterial species.

1.4.2 Minimum Inhibitory Concentration (MIC) Test

1.4.2.1 Standardized Susceptibility Test

The standardized method used today to test the susceptibility of an organism to a particular agent is the MIC test. The MIC is the lowest concentration of an antimicrobial agent that is required to inhibit the visible growth of an organism when compared to the appearance of growth in the control wells (NCCLS, 2001a,b,c,d;

Goldstein *et al*, 2002). To help determine the proper dosing of an antimicrobial agent, the MIC values are considered together with the pharmacokinetic parameters of the agent (Drlica and Schmitz, 2002). The MIC is currently an important parameter used for the development of dosing strategies for antimicrobial agents.

1.4.2.2 Problems Associated with MIC Testing

The MIC test has some disadvantages. A problem with the approach of basing dosing strategies on the MIC is that although the MIC is aimed at preventing proliferation of susceptible bacteria, it is not aimed at preventing the growth of resistant mutants that may be present in a population of cells (Drlica and Schmitz, 2002). Indeed, studies with mycobacteria show that the MIC does not reflect the ability of a quinolone agent to restrict the selection of resistant mutants (Sindelar *et al*, 2000; Gillespie *et al*, 2003). The amount of drug that inhibits visible growth on the MIC panel may not be the concentration that will eradicate an infection (Blondeau *et al*, 2001). The MIC may not eliminate all of the organisms and their potential mutants (upper portion of Figure 1.1) (Blondeau *et al*, 2001). As a result, the widespread use of some antimicrobial agents has been correlated with an increase in the number of resistant organisms.

The MIC test examines organisms at approximately 10^5 cfu/ml and in an infected patient, the bacterial numbers may be as high as 10^9 to 10^{10} cfu/ml (Gould and MacKenzie, 2002). For many organisms including *S. aureus*, the spontaneous mutation frequency has been reported as one mutation for every 10^6 to 10^8 cells (Zhao and Drlica, 2001). At inocula exceeding the spontaneous mutation frequency, resistance mutations are more likely to occur. To assess the susceptibility of an infecting isolate, one should test that organism at an inoculum exceeding the spontaneous mutation frequency.

Other concerns with MIC testing are also apparent. For example, the turbidity determinant is subjectively based, the doubling dilutions make results an approximation, and the observations are done only once after approximately 18-24 hr (Gould and MacKenzie, 2002). These concerns force one to seek a different approach to susceptibility testing of bacteria. Despite the limitations, MIC testing remains the primary method of antimicrobial susceptibility testing worldwide.

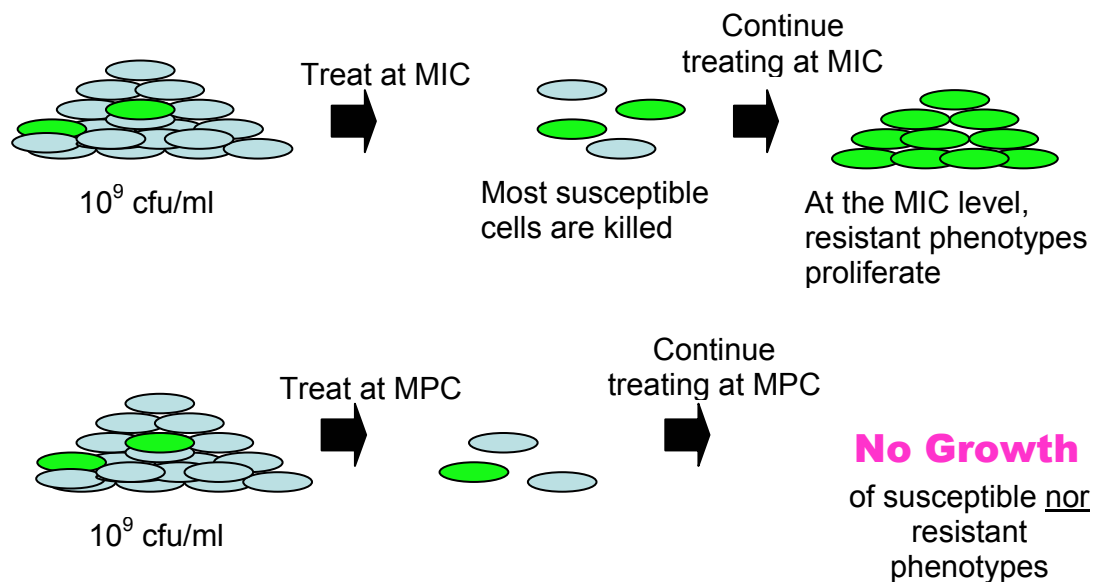
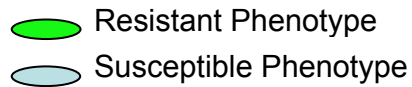


Figure 1.1: Schematic representation of the behavior of large bacterial populations when exposed to antimicrobial agents at the MIC and MPC.

The global dilemma of increasing resistance may be attributed partly to antimicrobial dosing strategies based on MIC testing. This increase in worldwide resistance is not as pronounced as one may expect considering the problems with MIC testing. This may be explained by other factors, such as the immune system which plays a significant role in the response to infection (Drlica and Schmitz, 2002). One should still be concerned, however, since the number of immunocompromised patients is increasing and one can no longer rely on the immune response to eliminate resistant cells that may be present in an infection (Drlica and Schmitz, 2002). Without a doubt, the traditional low-dose antimicrobial therapy, which was thought to have clinical efficacy, minimal toxicity, and lower cost, has exacerbated resistance problems (Blondeau *et al*, 2002b; Gould and MacKenzie, 2002).

As bacteria become increasingly resistant to the antimicrobials in use today, healthcare professionals are becoming more desperate to prevent this emerging resistance. It was originally thought that resistant mutants had a fitness deficit, however, there is evidence indicating that resistant mutants are just as hearty as susceptible cells (Gillespie *et al*, 2002). This finding raises concern as resistant mutants are able to withstand more extreme environments than originally thought. New treatment strategies to accompany antimicrobials should be considered to help stop the selection of resistant subpopulations and, hence, maintain the clinical efficacy of agents (Blondeau *et al*, 2001, 2002b).

1.4.3 Mutant-Prevention Concentration (MPC)

1.4.3.1 Theory

Because of the recognized problems associated with MIC testing, a novel measurement of susceptibility/resistance has been developed. The mutant-prevention concentration (MPC) is based on the concept that at a certain concentration of drug, emergence of resistant organisms will be prevented due to the killing of all organisms, including the potential mutants, by this increased concentration. Concentrations currently used for various infections destroy the majority of susceptible pathogens, but at the same time are often the very concentrations required to select a resistant pathogen (Zhao and Drlica, 2001). Instead of testing the organism at 10^5 cfu/ml, as the MIC test

is performed, the MPC approach utilizes organisms at an inoculum of $\geq 10^9$ cfu/ml. By increasing the inoculum, an environment conducive to the selection and proliferation of resistant mutants is created (Zhao and Drlica, 2001). Large bacterial inocula often exist as heterogeneous populations with a dominant phenotype mixed with mutant phenotypes (Drlica and Schmitz, 2002). The MPC has been described as a "...drug concentration threshold above which bacterial cells require the presence of two or more resistance mutations for growth." (Drlica and Schmitz, 2002). This is, indeed, true in the case of the fluoroquinolone and des-quinolone agents. For the quinolones, the spontaneous mutation frequency is approximately one mutation for every 10^7 cells (Zhao and Drlica, 2001). The MPC tests organisms at inocula exceeding this number of cells. Susceptibility testing above this frequency gives a concentration of drug above which no mutant should grow because a double mutation (requiring $10^7 \times 10^7 = 10^{14}$ bacterial cells) is extremely rare in nature and in human infectious diseases as patients would not have bacterial loads of this magnitude. The MPC is defined as the MIC of the least susceptible single cell mutant (Zhao *et al*, 2003). The MPC is, therefore, a concentration of drug that will prevent the growth of first-step resistant mutants present in a heterogeneous population of bacterial cells (lower portion of Figure 1.1).

Most of the relevant MPC data have been obtained with fluoroquinolones (Blondeau *et al*, 2001; Zhao and Drlica, 2001; Drlica and Schmitz, 2002; Hansen *et al*, 2003; Zhao *et al*, 2003). Non-quinolone agents have not been thoroughly studied with the MPC approach. It is important to test different families of antimicrobial agents to determine whether an increased inoculum plus the selective pressure of an agent have any effect on the concentration of drug required to prevent the growth of entire bacterial populations.

1.4.3.2 Application

The MPC values may be used in a similar way to the MIC values where the values are combined with the pharmacokinetic properties of antimicrobial agents and together new dosing strategies may be developed. The difference in the approach with the MPC values is that the new dosing strategies will be aimed at preventing resistance by killing both susceptible and resistant bacterial cells, whereas currently with the MIC values, dosing strategies may actually encourage growth of resistant subpopulations

(refer to Figure 1.1). The comparison of MIC and MPC values for an isolate, plus the plotting of these values on the pharmacokinetic curve of an antimicrobial agent, could provide additional information as to the appropriate dosing strategy of an agent. Therefore, the MPC may be an important parameter when considering prevention of resistance based on dosing strategies. To date, however, this strategy has been most extensively studied with fluoroquinolone agents.

1.4.4 Mutant-Selection Window (MSW)

1.4.4.1 Definition/Relationship to MIC and MPC Values

Following from the MPC, the concept of the mutant-selection window (MSW) was developed. The MSW is defined by the drug concentrations between the MIC and the MPC and includes the concentrations of drug that enrich resistant mutants (Zhao and Drlica, 2001). It has been previously shown that a *S. aureus* population can reduce its susceptibility to an agent while in the presence of drug concentrations that are within the MSW (Firsov *et al.*, 2003). Below the MIC, no mutant is selected because the selective pressure is insufficient as neither susceptible nor first-step resistant cells will be inhibited (Blondeau, 2003). Also, no mutant should grow above the MPC because that would require two mutations which, as stated previously in section 1.4.3.1, is rare. Within the MSW, susceptible cells are inhibited, however, resistant subpopulations may be selectively amplified (Blondeau, 2003).

1.4.4.2 Clinical Application

Current pharmacodynamic strategies result in drug concentrations that fall within the MSW for some antimicrobial compounds. The ultimate goal should be to avoid the MSW or to administer agents for as short time as possible while in the window (Zhao and Drlica, 2001). Because the MSW is measurable, antimicrobial agents can be compared to determine compounds that are better suited for administration at the MPC or above (Zhao and Drlica, 2001). Compounds better suited for therapy include those with a narrow or closed window (where the MIC is equal to the MPC). One may also avoid serum/tissue concentrations in the MSW by utilizing combination therapy or by adjusting the dosage regimen (Zhao and Drlica, 2001). The ideal dose for patients would be above the MPC, providing that it is clinically achievable, sustainable, and not likely to induce unfavorable toxicities (Zhao and

Drlica, 2001). During therapy, if the serum/tissue drug concentration is maintained above the MPC concentration, very few or no resistant mutants will be selectively amplified (Drlica and Schmitz, 2002). Thus, the MSW and, hence, MPC data, may be important when treating infections with antimicrobial agents against organisms with the potential to become resistant.

1.5 Summary

S. aureus is a problematic organism causing worldwide morbidity and mortality. As well, *S. aureus* rapidly develops resistance to antimicrobial agents. This, in turn, has compromised the use of many antimicrobial agents. Current dosing strategies may encourage the development of resistance. As new and effective agents are being developed, new dosing strategies should accompany these agents to prevent resistance to these novel agents.

The MPC is a novel test utilizing large bacterial populations with the presence of a selective antimicrobial agent. The MPC data may be clinically significant when treating patients with a *S. aureus* infection. As the prevalence of resistance increases, it is important that antimicrobial agents be considered for their propensity to selectively enrich resistant subpopulations (Zhao *et al*, 2003). The MPC data may be used to help determine agents less likely to select for resistant subpopulations. In addition, the MPC test provides a measurement of drug concentration required to eliminate first-step resistant mutants (Zhao *et al*, 2003). The objective is to use compounds that have low and narrow MSWs and that can be administered safely above the MPC (Zhao and Drlica, 2001). However, because dosing regimens for currently used agents were developed for achieving concentrations above the MIC, few of these agents are likely appropriate for maintaining concentrations above the MPC (Zhao and Drlica, 2002). The MPC is, therefore, useful in the development of new dosing strategies for novel agents or in helping to determine agents less likely to select for resistance based on the amount of time the drug concentration is within the MSW.

Clearly, it is important to determine if resistant subpopulations exist in wild type susceptible populations (as determined by MIC testing). Should such populations be found, the potential impact of inadvertently selecting for these resistant subpopulations with improper antimicrobial dosing strategies may be devastating for infected patients

on antimicrobial therapy. One may reduce the likelihood of selecting for resistance by treating patients with the proper dosage of drug and this dosage may be acquired with the help of the MPC data.

1.6 Objectives

The MPC approach has been shown to be significant for evaluating the emergence of resistance in *S. pneumoniae* organisms (Blondeau *et al*, 2001; Hansen *et al*, 2001; Tillotson *et al*, 2001; Blondeau *et al*, 2002b; Blondeau, 2003). The hypothesis I based my research upon is that the MPC approach is also important for evaluating the emergence of resistance in *S. aureus* organisms. As well, the MPC may provide valuable insight as to dosing strategies necessary to restrict the selection of resistant *S. aureus* subpopulations.

My first objective was to determine MPC values for MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates to different families of antimicrobial agents. From these data, one may compare the *in vitro* potency of the agents using MPC values. As well, one may compare the behavior of MSSA and MRSA isolates to different antimicrobial agents. Using MIC and MPC data, one may establish the MSW and, hence, determine agents least likely to select for resistant subpopulations.

My second objective was to study the susceptibility behavior of sequential *S. aureus* isolates. Scientists hypothesize that throughout antimicrobial therapy a patient may develop an infection that is resistant as a result of selective amplification of a resistant subpopulation. There are few reports proving that treatment with an antimicrobial agent may select resistant mutants, *in vivo* (Davidson *et al*, 2002; Anderson *et al*, 2003). MIC or MPC changes among multiple sequential organisms isolated from patients undergoing antimicrobial therapy was studied. Studies such as these may provide evidence that resistant subpopulations may proliferate as a result of antimicrobial selection *in vivo*.

2.0 MATERIALS AND METHODS

2.1 Standard Laboratory Methods

2.1.1 Isolate Collection and Identification

The *S. aureus* isolates used in this study were collected through the Clinical Microbiology Department at Royal University Hospital (RUH), Saskatoon, Saskatchewan, Canada. Individual isolates were obtained from February (2001) to September (2001), while sequential isolates were obtained from October (2001) to March (2002). Identification of *S. aureus* was in accordance with the Manual of Clinical Microbiology (Murray *et al*, 2003) determined by Latex agglutination (Staphaurex™) as recommended by Remel, Incorporated, 2002. The MRSA isolates were determined as such by the Oxacillin Screen Plate (Murray *et al*, 2003) or by PCR analysis of the *mecA* determinant gene (Predari *et al*, 1991).

S. pneumoniae isolates were collected between May (2002) and December (2002). Identification of *S. pneumoniae* was in accordance with the Manual of Clinical Microbiology (Murray *et al*, 2003) determined by traditional methods, including catalase testing, bile solubility, and optochin sensitivity. A novel method using the Slidex Pneumo-Kit by bioMerieux (in accordance with the instructions provided by the supplier) was also used to identify *S. pneumoniae* isolates.

2.1.2 Storage of the Bacterial Isolates

The isolates were obtained from Tryptic Soy Agar (TSA) slants (in house), TSA plates containing 5% sheep blood, and from frozen skim milk. Organisms identified as *S. aureus* or *S. pneumoniae* were streaked for isolated colonies onto a TSA plate containing 5% sheep blood and incubated in ambient air at 35-37°C for approximately 24 hr. One or two isolated colonies were selected from the TSA plate with a sterile wooden applicator stick and inoculated into 1.2 ml Corning cryovials containing 0.5 ml of skim milk. The vials were stored at -70°C.

2.1.3 Susceptibility Testing

There are a variety of methods by which to test the susceptibility of an isolate. The most common methods used today in clinical laboratories are the disk diffusion test (Kirby-Bauer), E-test, agar dilution test, and broth microdilution (Andersen, 2000). Broth microdilution and E-tests were performed in this study. Most healthcare professionals view broth microdilution as an accepted, reliable, and well-standardized method for susceptibility testing.

2.1.3.1 Broth Microdilution

MIC values were determined from the broth microdilution test in accordance with NCCLS guidelines (NCCLS, 2001a,b,c,d). Ninety-six well flat bottom microtitre plates were filled with 100 μ l of Mueller Hinton Broth (MHB) in each well of columns 2-12. Antimicrobial agent was serially diluted across the plate with column 1 containing the highest drug concentration and column 12 receiving no drug (growth control). Each isolate was standardized to a 0.5 McFarland suspension using a Colorimeter ($\sim 1.0 \times 10^8$ cfu/ml). The bacterial suspension was then diluted 1/100 with MHB ($\sim 1.0 \times 10^6$ cfu/ml). One hundred μ l of diluted cells were added to each well on the plate resulting in a final volume of 200 μ l. Purity of the bacterial suspension was confirmed by plating each sample onto a fresh TSA plate containing 5% sheep blood. The plates (both microtitre and blood agar) were incubated in ambient air at 35-37°C for 16-20 hr. The growth control wells (column 12) were examined prior to MIC determination to ensure organism viability. Also, the purity plates were examined prior to MIC determination to ensure the culture remained pure throughout the experiment. The American Type Culture Collection (ATCC) strain, *S. aureus* 29213, was used as a control to confirm accuracy of each MIC test (ATCC, Manassas, VA 20108 U.S.A). MIC control ranges for isolate 29213 are listed in Appendix D for the various antimicrobials that were tested by broth microdilution. The MIC value was recorded as the lowest drug concentration at which there was no visible growth of the organism. The antibiotics tested by broth microdilution included: azithromycin, cefazolin, cefotaxime, cefuroxime, cephalixin, cloxacillin, ciprofloxacin, garenoxacin, gatifloxacin, gemifloxacin, gentamicin, levofloxacin, moxifloxacin, rifampin, piperacillin/tazobactam, and vancomycin.

Broth microdilution for *S. pneumoniae* isolates was done similar to that described for *S. aureus* isolates. However, Todd Hewitt Broth (THB) was used, as was the *S. pneumoniae* control strain, ATCC 49619. All plates were incubated at 35-37°C in 5% CO₂ and 95% ambient air for 16-20 hr. Agents tested with *S. pneumoniae* by microdilution include azithromycin, clarithromycin, and erythromycin. Control ranges of *S. pneumoniae* 49619 for azithromycin, clarithromycin, and erythromycin are listed in Appendix D.

2.1.3.2 E-Test

E-tests were also performed to determine the clindamycin MIC value of isolates. E-test strips contain high antimicrobial concentrations at one end of the strip with decreasing drug concentrations proceeding towards the opposite end of the strip. Each organism was standardized to a 0.5 McFarland inoculum. *S. aureus* ATCC 29213 was used as a control to confirm the accuracy of the resulting MIC values. Sterile swabs were used to transfer each isolate from the 0.5 McFarland suspensions to TSA plates containing 5% sheep blood. Using forceps, E-test strips were carefully placed in the middle of each plate. The 0.5 McFarland suspensions of bacteria were tested for purity by placing sterile wooden applicator sticks into the tubes and streaking for isolated colonies onto TSA plates containing 5% sheep blood. The plates were then incubated at 35-37°C in ambient air for 18-24 hr. Results were recorded according to the corresponding location on the strip where the growth of the organism was inhibited. MIC values are the lowest drug concentration values where no bacterial growth occurred.

2.2 Mutant-Prevention Concentration (MPC)

The MPC values for *S. aureus* were determined with a protocol developed as part of this thesis work (Metzler *et al*, in press). The protocol was based on a MPC protocol published earlier for *S. pneumoniae* (Blondeau *et al*, 2001). *S. aureus* ATCC 29213 was used as a control strain.

2.2.1 Inoculum

Each *S. aureus* isolate was subcultured from thawed skim milk onto TSA plates containing 5% sheep blood using a sterile wooden applicator stick and incubated in ambient air at 35-37°C for approximately 24 hr. A sterile swab was used to collect as

much bacteria as possible from each plate and then sub-cultured onto three fresh TSA plates containing 5% sheep blood (two plates were entirely covered with the bacteria and one plate was streaked for isolated colonies). These plates were then incubated in ambient air for approximately 24 hr. Sterile swabs were used to transfer the contents of the plates to sterile bottles containing 100 ml of MHB. One hundred μ l were taken from bottles and streaked onto plates containing various concentrations of antimicrobial agent. Spectrophotometer readings were recorded for each sample by adding 2 ml of the inoculum to a clean cuvette and placing it in the spectrophotometer. Organisms with a spectrophotometer reading <1.0 were re-grown until an inoculum with a spectrophotometer reading of ≥ 1.0 was observed. Previously, it was determined that readings ≥ 1.0 resulted in inocula of $\geq 10^9$ cfu/ml for *S. aureus* organisms (in house observation). All plates were incubated for 48 hr in ambient air (35-37°C) and screened for growth at both 24 and 48 hr. The MPC was recorded as the lowest drug concentration that had no visible bacterial growth on the plate.

The MPC procedure for *S. pneumoniae* isolates varied slightly from the MPC procedure used for *S. aureus* isolates. *S. pneumoniae* ATCC 49619 was tested in each MPC test performed with *S. pneumoniae* isolates. The MPC protocol used for *S. pneumoniae* followed the format described in Blondeau *et al*, 2001. Briefly, from a fresh overnight culture, *S. pneumoniae* isolates were plated onto eight plates and incubated at 35-37°C in 5% CO₂ and 95% atmospheric air for 24 hr before inoculation into 500 ml of THB. After a 24 hr incubation of the 500 ml inoculum at 35-37°C in 5% CO₂ and 95% atmospheric air, each isolate was centrifuged in an Avanti J-E centrifuge (Beckman Coulter) at 5000 X g for 30 min. Pellets were resuspended in 3 ml of THB and 200 μ l were plated onto TSA plates containing various concentrations of drug. Plates were incubated in 5% CO₂ and 95% atmospheric air at 35-37°C for 48 hr with observations recorded at both 24 and 48 hr.

2.2.2 Antimicrobial Plates

Initial concentrations used in the experiment consist of one doubling dilution below the MIC, the MIC, and five doubling dilutions above the MIC. Upon repeat testing, the range of drug concentrations varied depending on the behavior of the organism (if the organism did not grow on any antimicrobial plates, the MIC was

repeated and the MPC range was lowered; if the organism grew at the highest drug concentration plate, the MPC range was extended and the experiment was repeated at a higher range). Antimicrobial agents were prepared as described in Appendix C. TSA was prepared from powder form according to the manufacturer's descriptions as described in Appendix A. The agar was kept at 55°C in a water bath until it was ready to pour. To determine the amount of agent to add, I used the following formula:

$$C_1V_1=C_2V_2 \quad (2.1)$$

Where C_1 was equal to the stock concentration of antimicrobial agent, C_2 was equal to the desired concentration, V_2 was equal to the total volume, and V_1 was equal to the volume of antimicrobial agent that must be added to obtain the desired drug concentration. Approximately 20 ml of agar containing drug were poured into each sterile petri plate and each plate was labeled appropriately with the name of the agent and its concentration. Addition of 5% sheep blood to the TSA was necessary for the proper growth of *S. pneumoniae* isolates.

2.2.3 Viable Counts

Viable counts were performed on selected organisms for each round of MPC tests. Dilutions of 10^{-7} , 10^{-8} , and 10^{-9} were made from the bacterial suspension and 100 μ l of each dilution were plated onto triplicate TSA plates containing 5% sheep blood. The plates were incubated for 24 hr at 35-37°C in ambient air. Colony counts were performed on each plate. Calculations were then performed to determine colony forming units per ml. This was done by multiplying the average of three plates by the reciprocal of the dilution factor and then multiplying by 10 for results in colony forming units per ml (cfu/ml).

2.3 Characterization of Bacterial Isolates

2.3.1 Polymerase Chain Reaction (PCR)

2.3.1.1 Primer Preparation

Primers used in this study are listed in Table 2.1. Each primer, including the upstream and the downstream primer, were dissolved at a concentration of 100 μ M by

Table 2.1: Primers used in this study.

Resistant Gene		Sequence	nmol	% GC	PCR Product Size (bp)
vanA ¹	Upstream	5' GGGAAAACGACAATTGC 3'	53	47	732
	Downstream	5' GTACAATGCGGCCGTTA 3'	56	52	
vanB ¹	Upstream	5' ATGGGAAGCCGATAGTG 3'	58	52	635
	Downstream	5' GATTTCGTTCTCGACC 3'	73	52	
vanC ¹	Upstream	5' GGTATCAAGGAAACCTC 3'	54	47	822
	Downstream	5' CTTCCGCCATCATAGCT 3'	55	52	
vanC2/C3 ²	Upstream	5' CGGGGAAGATGGCAGTAT 3'	45	56	484
	Downstream	5' CGCAGGGACGGTGATTTT 3'	36	56	
vanD ³	Upstream	5' TAAGGCGCTTGCATATACCG 3'	60	50	461
	Downstream	5' TGCAGCCAAGTACCGGTAA 3'	49	50	
vanE ⁴	Upstream	5' TGTGGTATCGGAGCTGCAG 3'	68	58	513
	Downstream	5' GTCGATTCTCGCTAATCC 3'	62	50	
vanG ⁵	Upstream	5' CGGTTGTGCCGTACTTGGC 3'	45	63	810
	Downstream	5' GGGTAAAGCCATAGTCTGGGGC 3'	55	59	
ermA ⁶	Upstream	5' GTTCAAGAACAATCAATACAGAG 3'	51	35	421
	Downstream	5' GGATCAGGAAAAGGACATTTTAC 3'	51	39	
ermB ⁷	Upstream	5' GAAAAGGTACTAAACCAAATA 3'	48	29	639
	Downstream	5' AGTAACGGTACTTAAATTGTTTAC 3'	64	29	
ermC ⁶	Upstream	5' GCTAATATTGTTTAAATCGTCAATTCC 3'	61	30	572
	Downstream	5' GGATCAGGAAAAGGACATTTTAC 3'	64	39	
mefA ⁷	Upstream	5' ACTATCATTAATCACTAGTGC 3'	48	33	348
	Downstream	5' TTCTTCTGGTACTAAAAGTGG 3'	40	38	
msrA/msrB ⁸	Upstream	5' GCAAATGGTGTAGGTAAGACAACT 3'	52	42	400
	Downstream	5' ATCATGTGATGTAAACAAAAT 3'	46	24	
mecA ⁹	Upstream	5' GGGATCATAGCGTCATTATTC 3'	85	43	526
	Downstream	5' AACGATTGTGACACGATAGCC 3'	51	48	

¹Dutka-Malen *et al* (1995)²Kariyama *et al* (2000)³Perichon *et al* (1997)⁴Fines *et al* (1999)⁵McKessar *et al* (2000)⁶Lim *et al* (2002)⁷Hoban *et al* (2001)⁸Wondrack *et al* (1996)⁹Predari *et al* (1991)

adding the appropriate volume of ½TE Buffer (preparation of buffer described in Appendix A) to dried primer which had been stored at -20°C. Each tube was then vortexed. Dissolved primer was diluted to 20 µM with ½TE Buffer. Equal volumes of the upstream and the downstream primer were then added to a single tube and vortexed. Primer solutions were stored at -20°C.

2.3.1.2 Setting up the PCR Reaction Tubes

Bacterial isolates were thawed and plated on a TSA plate (containing 5% sheep blood) as described in section 2.1.2. From the blood plate, a visible loop of bacteria was added to 100 µl of InstaGene™ Matrix. For isolates tested for *van* genes, 10 µl of specimen were taken directly from thawed saline and added to 100 µl of InstaGene™ Matrix. Each specimen was boiled for 10-12 min and then centrifuged for ~45 sec at 16,000 X g. Three µl of primer were added to each tube of PuReTaq Ready-To-Go PCR Beads. A total of 19.5 µl of sterile distilled water were added to each PCR reaction tube. Finally, 2.5 µl of supernatant from each specimen were added to the appropriate PCR reaction tubes resulting in a final volume of 25 µl. Each PCR reaction tube was vortexed vigorously until the PuReTaq Ready-To-Go PCR bead was completely dissolved. During preparation of samples, latex gloves were worn at all times.

2.3.1.3 Thermocycler Settings

Settings for the thermocycler varied, depending on the primers that were used. Table 2.2 displays the thermocycler settings that were used for each primer set. For *ermA*, *ermC*, and *msrA/msrB*, two sets of parameters were used for PCR. After the final extension for each set of parameters, the samples were kept at 4°C until electrophoresis.

2.3.1.4 Agarose Gel Electrophoresis

PCR samples were run on a 1% agarose gel prepared as described in Appendix A. Six µl of Tracking Dye were added to each PCR reaction tube. For the marker, 2 µl of a 123 bp ladder were added to 12 µl of sterile distilled water and 4 µl of Tracking Dye. Each tube was gently mixed. Approximately 20 µl were loaded into designated lanes. The gel was run at 117 volts for 20-30 min.

Table 2.2: Thermocycler conditions for the primers used in this study.

Primers	Initial Denaturation		Denaturation		Annealing		Elongation		# Cycles	Final Elongation	
	Temp ¹	Time (min)	Temp	Time (sec)	Temp	Time (sec)	Temp	Time (sec)		Temp	Time (min)
<i>ermA</i> ²	94	2	94	60	53	60	72	60	30	72	10
<i>ermA</i> ³	95	5	94	30	54	30	72	60	35	72	5
<i>ermB</i> ²	94	2	94	60	53	60	72	60	30	72	10
<i>ermC</i> ²	94	2	94	60	53	60	72	60	30	72	10
<i>ermC</i> ³	95	5	94	30	54	30	72	60	35	72	5
<i>mefA</i> ²	94	2	94	60	53	60	72	60	30	72	10
<i>msrA/msrB</i> ²	94	2	94	60	53	60	72	60	30	72	10
<i>msrA/msrB</i> ⁴	95	3	93	30	55	120	72	90	35	72	10
<i>mecA</i> ⁵	95	4	94	35	56	35	72	40	35	72	6
<i>vanA</i> ⁶	94	2	94	1	54	1	72	60	30	72	10
<i>vanB</i> ⁶	94	2	94	1	54	1	72	60	30	72	10
<i>vanC</i> ⁶	94	2	94	1	54	1	72	60	30	72	10
<i>vanC2/C3</i> ⁷	94	5	94	30	53	30	72	30	35	72	10
<i>vanD</i> ⁷	94	5	94	30	53	30	72	30	35	72	10
<i>vanE</i> ⁷	94	5	94	30	53	30	72	30	35	72	10
<i>vanG</i> ⁷	94	5	94	30	53	30	72	30	35	72	10

¹Temp=Temperature

²Thermocycler conditions derived from Hoban *et al* (2001)

³Thermocycler conditions derived from Lim *et al* (2002)

⁴Thermocycler conditions derived from Wondrack *et al* (1996)

⁵Thermocycler conditions derived from Predari *et al* (1991)

⁶Thermocycler conditions derived from Dutka-Malen *et al* (1995)

⁷Thermocycler conditions derived from Kariyama *et al* (2000); Perichon *et al* (1997); Fines *et al* (1999); McKessar *et al* (2000)

2.3.2 Electron Microscopy (EM)

2.3.2.1 Preparation of Samples

The *S. aureus* sample for electron microscopy (EM) was grown up continually under the selective pressure of the antimicrobial agent. Isolate #41m was recovered from an MPC agar plate containing 4 µg/ml of vancomycin. MHB containing 4 µg/ml of vancomycin was then inoculated with #41m. The inoculated tube was placed in a shaking water bath at 37°C for 48 hr. An additional tube containing MHB was inoculated with #41 (parent isolate of #41m) and was then placed in the shaking water bath at 37°C for 24 hr. Five hundred µl were taken out of each tube, added to 1.5 ml eppendorf tubes, and the cells were spun down at 16,000 X g for 1 min. Broth was decanted with a Pasteur pipette and 300 µl of 2% Glutaraldehyde were quickly added to each tube.

2.3.2.2 Saskatoon Health Region Protocol

The remaining steps required for EM were performed by Karen Slattery (Department of Pathology, Saskatoon Health Region, SHR). Briefly, the samples were fixed for 1 hr in 2% glutaraldehyde and then were washed twice (15 min each time) in 0.1M sodium cacodylate buffer. The samples were then post-fixed in 2% osmium tetroxide for 1 hr. Dehydration of the samples was carried out through three 5 min rinses in increasing concentrations of ethanol (50, 70, and 95%). The samples then went through three 5 min rinses of propylene oxide and followed by a 50:50 mixture of propylene oxide and epon araldite, the embedding media. The samples were then embedded in pure epon araldite in BEEM capsules and hardened overnight at 80°C. The blocks were sectioned at 1 µm using a Reichert ULTRACUT E then stained with 1% Toluidine Blue, cover slipped and screened using a light microscope. Ultra thin sections were cut at 60-90 nm then stained with 3% uranyl acetate and Reynold's lead citrate. The samples were examined using a Zeiss EM 10C electron microscope and micrographs were printed of areas of interest.

2.3.2.3 EM Analysis

Various fields of view containing many cells were photographed at 40,000X. Blind measurements were taken at the north, east, south, and west sides of each cell wall, where visible. The prints were 2.4X the negative, therefore, the magnification was

multiplied by 2.4 to result in a final magnification of 96,000X. The initial measurements were recorded in millimeters and were divided by 96,000 and multiplied by 1000 to convert the results to μm . From there, the measurements were converted to angstroms by multiplying by 10,000. Statistical analysis was performed between cell wall measurements taken from the parent susceptible population (#41) and from the MPC-recovered population (#41m).

2.3.2.3.1 Statistical Analysis

A right-tailed hypothesis test according to Mann (1998b) was set up to determine if the mean of the cell wall measurements for #41m was significantly greater than the mean of cell wall measurements for #41. The average of both population sets and standard deviations were determined. The calculation used for standard deviation was as follows:

$$s = \sqrt{s^2} \quad (2.2)$$

$$s^2 = \frac{\sum(x-\mu)^2}{n-1} \quad (2.3)$$

Where 'x' was equal to the measurement of the cell wall, ' μ ' was equal to the mean of the cell wall size for the sample population, and 'n' was equal to the number of measurements taken for the sample population. The number of measurements for each population was ≥ 30 , therefore, the normal distribution curve was used. The critical value (Z_c) is dependent on the significance level (α) of the test. The sample test value (Z_t) was calculated as follows:

$$Z_t = \frac{\mu_{\#41m} - \mu_{\#41}}{s_{\mu_{\#41} - \mu_{\#41m}}} \quad (2.4)$$

$$\text{Where } s_{\mu_{\#41} - \mu_{\#41m}} = \sqrt{\left[\frac{(S_{\#41})^2}{n_{\#41}} + \frac{(S_{\#41m})^2}{n_{\#41m}} \right]} \quad (2.5)$$

If the test value exceeds the critical value, I reject the null hypothesis (H_0) which stated that the cell wall thicknesses are not significantly different.

2.3.3 Sequencing

2.3.3.1 Preparation From a Gel Sample

A PCR-amplified DNA fragment was taken from a gel and prepared for sequencing. This was done by cutting out the desired band from the gel while using an ultraviolet light. The band was placed in a tube with 500 μ l of sterile distilled water and kept at 4°C overnight. Two PCR beads (described in section 2.3.1.2) were then placed in a single tube. A total of 6 μ l of primer were added to the tube, plus 33 μ l of sterile distilled water. After vigorous vortexing, 5 μ l of the overnight sample containing the gel were added to the tube.

Also, a sample was prepared from a fresh overnight culture. This was done by boiling the cells for 10 min from a fresh culture. Then, 5 μ l of sample were placed into a tube containing two PuReTaq Ready-To-Go PCR beads. As mentioned above, 6 μ l of primer plus 33 μ l of sterile distilled water were also added to the tube.

The samples (both the sample from a gel and the sample from a fresh culture) were placed in the thermocycler and the settings used were those that were used for the PCR experiment. After the thermocycler had completed its cycles, 12.5 μ l of sample, plus 6 μ l of tracking dye were loaded into the gel. Leftover sample was saved for further preparation for sequencing with the Wizard Kit. The samples were then run on a 1% agarose gel for 30 min at 117 volts. The gel was then analyzed with the Gel-Doc 1000 Illuminator system to ensure purity as indicated by a distinct, single band.

2.3.3.2 Wizard Kit Protocol

The protocol used was based on the directions supplied by the manufacturer. A total of 100 μ l of Wizard Direct Purification Buffer™ were added to the sample and vortexed briefly. One ml of Wizard Purification Resin™ was added to the tube and the tube was vortexed briefly three times during a 1 min period. The sample was then put through a mini column into an empty tube. Following the sample, two ml of 80% isopropanol were put through the mini column. The sample was then centrifuged for 2 min. Fifty μ l of water were added to the sample. After 1 min, the sample was centrifuged for 20-60 sec. The sample was then quantified. This was done by running 10 μ l of the sample in one lane and a mass ladder with known DNA concentrations in a neighboring lane of the gel. A visual comparison of the intensity of the sample band to

the intensities of the known concentrations of the bands in the mass ladder was performed.

2.3.3.3 Sequencing Procedure

The sequencing procedure was performed by Jennifer Beck in the DNA Sequencing Laboratory at the National Research Council. An upstream primer was sent at a concentration of 3.2 pmole/ μ l along with the sample at a concentration of 10 ng/ μ l.

2.3.4 Pulsed Field Gel Electrophoresis (PFGE)

2.3.4.1 Preparation of Samples

A few colonies from a pure culture of each *S. aureus* isolate were chosen from a TSA plate containing 5% sheep blood and inoculated into 5 ml of MHB. The samples were then placed in a shaking water bath at 37°C for 16-18 hr. Each sample was then centrifuged at 16,000 X g for 1 min. Each pellet was resuspended in 150 μ l of Cell Suspension Buffer (CSB) (described in Appendix A).

2.3.4.2 Casting Plugs

Preparation of a 2% low melting point (LMP) agarose in CSB was performed for the casting plugs (Appendix A). Two μ l of Lysostaphin at 1 mg/ml were added to each tube followed by the immediate addition of 150 μ l of 2% LMP agarose. The tube was mixed well and then the contents were added to the casting mold. The casting plugs were left to solidify for 30 min at room temperature and 30 min at 4°C.

2.3.4.3 Lysis of Cells in LMP Agarose

Lysis Buffer (Appendix A) was made up fresh and 750 μ l were added to a new tube for each sample. The plugs were gently transferred from the mold to their corresponding labeled tube containing the Lysis Buffer. Each plug was then incubated at 37°C for 1 hr. The Lysis Buffer was then aspirated off with a 1 ml pipette. Plugs were then transferred to microfuge tubes. Proteinase K (20 mg/ml) was diluted in Proteinase K Buffer (final concentration equaling 50 μ g/ml) and 750 μ l of this solution were added to each microfuge tube containing the plugs. The tubes were then incubated in a shaking water bath at 50°C for 0.5 hr to overnight.

2.3.4.4 Washing LMP Plugs

After removing the tubes from the 50°C water bath, the Proteinase K solution was aspirated off. The plugs were rinsed once with 1 ml of Wash Buffer (Appendix A).

Three additional washes with 1 ml of fresh Wash Buffer each time was performed for at least 30 min for each wash. The washes were conducted at room temperature. The Wash Buffer was removed after the final wash and 1 ml of fresh Wash Buffer was added for long term storage of the plugs.

2.3.4.5 Restriction Enzyme Digestion of LMP Plugs

Approximately one third of each plug was cut on a clean surface and placed into a new 1.5 ml microfuge tube. Four hundred and fifty µl of 1X Buffer A were added to each tube and the contents of the tube were allowed to sit for 10 min to equilibrate each sample. *SmaI* was used to restrict the DNA. A total of 25 Units were used per sample. The volume required was calculated as follows:

$$\frac{\text{Total enzyme required (Units)}}{\text{SmaI Concentration (Units/}\mu\text{l)}} = \text{Volume required} \quad (2.6)$$

Buffer A was removed from the plugs and 150 µl of Buffer A/*SmaI* mixture were added to each tube. The tubes were then incubated at 25°C overnight.

2.3.4.6 Casting Agarose Gel

The samples were run on a 1% pulsed field agarose gel prepared as described in Appendix A. Approximately 2 L of 0.5X TBE was added to the Contour Clamped Homogenous Electric Field (CHEF) chamber. The cooling module was set at 14°C and the pump was turned on to allow the buffer to cool. The enzyme/buffer solution was aspirated from each sample and the plugs were melted in a water bath set at 65-70°C for 10-15 min. A Lambda DNA Ladder was run in at least one lane and was used to help determine sizes of the resulting bands. Thirty µl of each sample were loaded into the designated lanes. The gel was then placed into the electrophoresis chamber. Conditions for electrophoresis are given in Appendix B.

2.3.4.7 Staining and Documentation of the Gel

After 18 hr under pulsed field conditions, the gel was placed into a container with ethidium bromide (ETBr) (see Appendix A). The gel was stained for 20-30 min on a slowly rocking surface while prohibiting the exposure of the gel to light. The ETBr solution was then drained and destaining was performed by adding 500 ml of distilled

water to the container and placing it back on the rocking surface, again covered, for 30 min. The gel was analyzed on the Gel Doc 1000 Illuminator (See Appendix E).

2.4 Miscellaneous Experiments

2.4.1 Overcrowding

The overcrowding experiment was performed with a protocol similar to the MPC procedure for *S. aureus* described in section 2.2. However, in the overcrowding experiment, volumes of inoculum to be added to the plates varied between 25 μ l and 2 ml. For each different volume of inoculum, seven plates containing various drug concentrations were used. Viable counts were performed and cfu added to each plate were determined by dividing cfu/ml by the volume added to the plate.

2.4.2 Cefinase

The production of β -lactamase was determined by the Cefinase nitrocefin disk test (See Appendix E). *S. aureus* isolates were subbed onto TSA plates containing 5% sheep blood and incubated in ambient air at 35-37°C for approximately 24 hr. A positive and a negative control were included in each experiment. The positive control was *S. aureus* ATCC 29213. The negative control was *Haemophilus influenzae* ATCC 10211. Each cefinase disc was placed on a clean, flat surface and using a sterile Pasteur pipette, one drop of sterile distilled water was gently placed on each disc. Isolated colonies were acquired from a fresh culture plate with a sterile wooden applicator stick and placed onto a Cefinase disc. Appearance of a reddish color indicated the organism was a β -lactamase producer.

3.0 RESULTS

3.1 *S. aureus* Results Against Fluoroquinolones

A total of 122 MSSA and 22 MRSA clinical isolates were tested against gatifloxacin, gemifloxacin, levofloxacin and moxifloxacin. The distribution of MIC and MPC values for the MSSA strains are summarized in Table 3.1. Table 3.2 shows the MIC and corresponding MPC values for the 22 MRSA strains against the same four fluoroquinolones. The calculation of MIC_{50/90}, MPC_{50/90}, time above MPC measurements and other pharmacological related calculations are summarized in Table 3.3.

The MIC₉₀ represents the drug concentration that inhibited growth of 90% of isolates tested by standardized susceptibility testing (Blondeau *et al*, 2003a). Often, MIC data are considered based on the MIC₉₀ values because the MIC₉₀ is more convenient than analyzing each MIC value independently. MIC₉₀ data are also used globally as a marker for comparing *in vitro* antimicrobial potency. The MPC₉₀ is a concentration value that prevents growth of 90% of isolates tested by the MPC method against a particular antimicrobial agent. Like the MIC₉₀, this value is a more convenient way of interpreting MPC data as opposed to analyzing each value independently. The MIC₅₀ indicates the drug concentration that inhibited growth of 50% of isolates tested by the MIC. The MPC₅₀ is the drug concentration that inhibited growth of 50% of isolates tested by the MPC. The MIC mode is the MIC value that occurred most frequently in a standard MIC susceptibility test involving many isolates. Similarly, the MPC mode is the MPC value that occurred most frequently in MPC testing of the same isolates.

By broth microdilution testing of MSSA strains, MIC₉₀ values for gemifloxacin and moxifloxacin were 0.063 µg/ml (mode=0.031 µg/ml), as compared to 0.25 µg/ml for gatifloxacin (mode=0.063 µg/ml) and 0.25 µg/ml for levofloxacin (mode=0.25 µg/ml). Four isolates had MICs ≥ 2 µg/ml for gatifloxacin as compared to two for gemifloxacin, two for levofloxacin and one for moxifloxacin. By MPC (Table 3.1), the

Table 3.1: The minimum inhibitory concentration (MIC) and mutant-prevention concentration (MPC) of four fluoroquinolones against 122 clinical isolates of methicillin-susceptible *S. aureus*.

	No. of isolates with the following MIC (µg/ml) values									
Fluoroquinolone	≤0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	≥8
Gatifloxacin		18	56	30	11	3		2	2	
Gemifloxacin	37	47	29	5	1	1			2	
Levofloxacin			10	44	59	4	3		2	
Moxifloxacin	34	44	36	4	2		1	1		
	No. of isolates with the following MPC (µg/ml) values									
Gatifloxacin (n=119)				24	61	21	5	3	1	4
Gemifloxacin			27	66	16	4	4	1	4	
Levofloxacin (n=121)					3	93	15	2	7	1
Moxifloxacin			2	89	20	3	5	1	2	

Table 3.2: Comparative minimum inhibitory concentration (MIC) and mutant-prevention concentration (MPC) ($\mu\text{g/ml}$) values of four fluoroquinolones against 22 methicillin-resistant strains of *S. aureus*.

Organism #	Gatifloxacin		Gemifloxacin		Levofloxacin		Moxifloxacin	
	MIC	MPC	MIC	MPC	MIC	MPC	MIC	MPC
11	0.031	0.25	≤ 0.008	0.063	0.125	0.5	0.016	0.125
12	4	16	2	16	8	64	1	8
13	4	16	2	16	8	64	1	8
14	4	16	2	32	8	64	2	8
15	0.25	0.25	≤ 0.008	0.125	0.125	0.5	0.031	0.125
16	>8	32	4	32	16	64	2	8
17	0.063	0.25	≤ 0.008	0.125	0.5	0.5	0.016	0.25
18	4	16	4	16	16	64	0.016	8
19	8	32	8	512	>16	128	0.016	16
36	4	16	2	32	8	64	2	8
37	0.063	0.25	≤ 0.008	0.125	0.063	0.5	0.016	0.125
38	0.063	0.25	0.016	0.125	0.125	8	0.016	1
40	8	16	4	32	8	64	2	8
41	0.063	0.25	≤ 0.008	0.125	0.125	0.5	0.125	0.25
42	8	32	>8	512	>16	128	4	16
43	8	16	2	16	8	64	2	8
44	0.063	0.25	≤ 0.008	0.063	0.125	0.5	0.25	0.125
45	0.25	0.25	≤ 0.008	0.063	0.25	0.5	0.031	0.031
46	8	0.25	>8	256	>16	128	8	16
47	8	32	4	32	8	64	4	8
49	4	16	2	16	8	64	2	8
50	4	16	4	16	0.16	64	2	8

Table 3.3: Fluoroquinolone activity for clinical isolates of *S. aureus*.

	Gatifloxacin ¹				Gemifloxacin ²				Levofloxacin ³				Moxifloxacin ⁴			
	MSSA		MRSA		MSSA		MRSA		MSSA		MRSA		MSSA		MRSA	
MIC ₅₀ ^a (μg/ml)	0.063		4		0.031		2		0.25		8		0.031		1	
MIC ₉₀ ^a (μg/ml)	0.25		8		0.063		8		0.25		>16		0.063		4	
MPC ₅₀ ^b (μg/ml)	0.25		16		0.125		16		0.5		8		0.125		8	
MPC ₉₀ ^b (μg/ml)	1		32		0.5		256		1		128		0.25		16	
C _{max} ^c (μg/ml)	4.2		4.2		1.6		1.6		5.7		5.7		4.5		4.5	
Half-life (h) ^c	8-10		8-10		7-8		7-8		5-7		5-7		12-14		12-14	
Time (h) >MIC ₅₀ ^c	>24		~2		>24		0		>24		0		>24		>24	
Time (h) >MIC ₉₀ ^c	>24		0		~24		0		>24		0		>24		~3	
Time (h) >MPC ₅₀ ^c	>24		0		~18		0		~24		0		>24		0	
Time (h) >MPC ₉₀ ^c	~12		0		~9		0		~18		0		>24		0	
AUC ₀₋₂₄ (μg/ml)	51.3		51.3		8.4		8.4		48		48		47.5		47.5	
	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free
AUC ₀₋₂₄ /MIC ₅₀ ^c	814.3	651.4	12.8	10.2	270.9	108.4	4.2	1.7	192	142.1	6	4.4	1532.3	965.4	47.5	29.9
AUC ₀₋₂₄ /MIC ₉₀ ^c	205.2	164.2	6.4	5.1	133.3	53.3	1.1	0.4	192	142.1	3	2.2	753.9	474.9	11.9	7.5
AUC ₀₋₂₄ /MPC ₅₀ ^c	205.2	164.2	3.2	2.6	67.2	26.9	0.5	0.2	96	71	0.8	0.6	380	239.4	5.9	3.7
AUC ₀₋₂₄ /MPC ₉₀ ^c	51.3	41	1.6	1.3	16.8	6.7	0.03	0.01	48	35.5	0.4	0.3	190	119.7	2.9	1.8
C _{max} /MIC ₅₀ ^c	66.7	53.4	1.1	0.9	51.6	20.6	0.8	0.3	22.8	16.9	0.7	0.5	145.2	91.5	4.5	2.8
C _{max} /MIC ₉₀ ^c	16.8	13.4	0.5	0.4	25.4	10.2	0.2	0.08	22.8	16.9	0.4	0.3	71.4	44.9	1.1	0.69
C _{max} /MPC ₅₀ ^c	16.8	13.4	0.3	0.2	12.8	5.1	0.1	0.04	11.4	8.4	0.09	0.07	36	22.7	0.6	0.38
C _{max} /MPC ₉₀ ^c	4.2	3.4	0.1	0.08	3.2	1.3	0.006	0.002	5.7	4.2	0.04	0.03	18	11.3	0.3	0.19
^a MIC ₅₀ ;MIC ₉₀ drug concentration (μg/ml) at which 50% and 90%, respectively, of isolates are inhibited.																
^b MPC ₅₀ ;MPC ₉₀ drug concentration (μg/ml)at which no mutants were recovered from 50% and 90% of isolates, respectively.																
^c Time(h)>MIC(MPC) _{50/90} -time serum drug concentrations remain above the MIC (MPC) for 50 and 90% of the isolates tested																
^c AUC ₀₋₂₄ /MIC(MPC) _{50/90} -area under the curve after 24 hours over the MIC (MPC) for 50 and 90% of the isolates tested																
^c C _{max} /MIC (MPC) _{50/90} -maximum serum concentration over the MIC (MPC) for 50 and 90% of the isolates tested																
¹ 20% protein binding (Nakashima <i>et al</i> , 1995).																
³ 26% protein binding (Fish and Chow, 1997).																
² 60% protein binding (Allen <i>et al</i> , 2000).																
⁴ 37% protein binding (Stass and Proeve, 1999).																

rank order of potency was moxifloxacin (mode 0.125 µg/ml; MPC₉₀ 0.25 µg/ml) > gemifloxacin (mode=0.125 µg/ml; MPC₉₀ 0.5 µg/ml) > gatifloxacin (mode=0.25 µg/ml; MPC₉₀ 1 µg/ml) = levofloxacin (mode 0.5 µg/ml; MPC₉₀ 1 µg/ml). Eight isolates had MPCs \geq 2 µg/ml against gatifloxacin as compared to five for gemifloxacin, ten for levofloxacin and three for moxifloxacin.

The determination of the MIC₉₀ or MPC₉₀ values was additive starting from the lowest MIC or MPC value to the highest. As such, a single or a few strains may change the MIC₉₀ or MPC₉₀ percent value by one doubling drug dilution. Such was the case with this study. For example, by MIC testing, gatifloxacin concentrations of 0.25 µg/ml inhibited 94% of the isolates tested, however, the drug concentration 0.125 µg/ml inhibited a large percentage of isolates as well (86%). For the other three fluoroquinolones, concentrations one doubling dilution below the MIC₉₀ inhibited <70% of isolates tested.

By MPC testing, a drug concentration of 1 µg/ml inhibited 91% of isolates and 0.5 µg/ml inhibited 87% of the isolates tested for gatifloxacin. Similarly, the drug concentration of 0.5 µg/ml inhibited 93% of isolates tested with gemifloxacin and 0.25 µg/ml inhibited 89% of the isolates tested. For levofloxacin and moxifloxacin, concentrations one doubling dilution below the MPC₉₀ inhibited 78% and 74% of isolates tested, respectively.

The MIC and MPC values for the 22 MRSA strains are shown in Table 3.2. As well, the calculations of MIC_{50/90}, MPC_{50/90}, and various pharmacological calculations with the MRSA strains are shown in Table 3.3. Seven isolates had MICs to gatifloxacin \geq 8 µg/ml, seven at 4 µg/ml, and the remainder (eight isolates) at \leq 0.25 µg/ml. For gemifloxacin, three isolates had MICs \geq 8 µg/ml, five at 4 µg/ml, six at 2 µg/ml, and the remainder (eight) at \leq 0.016 µg/ml. Thirteen isolates had MICs to levofloxacin \geq 8 µg/ml and the remainder (nine) at \leq 0.5 µg/ml. Only one isolate had an MIC to moxifloxacin at 8 µg/ml, two at 4 µg/ml, and the remainder were at 2 µg/ml (seven isolates) or less (12 isolates) – ten with MICs \leq 0.25 µg/ml.

The MPC values for MRSA strains were considerably higher than MIC values against all four agents: 13 isolates had MPCs \geq 16 µg/ml for gatifloxacin; for gemifloxacin, 14 isolates had MPCs \geq 16 µg/ml (3 at \geq 256 µg/ml); for levofloxacin, 15

isolates had MPCs ≥ 8 $\mu\text{g/ml}$ (14 at ≥ 64 $\mu\text{g/ml}$); for moxifloxacin, 14 isolates had MPCs ≥ 8 $\mu\text{g/ml}$ (three at 16 $\mu\text{g/ml}$). Organisms with elevated MPCs to one agent had elevated MPCs to all four compounds.

Previous published literature has argued that the time the drug concentration remains in excess of the MPC may be important for restricting the development of resistance (Blondeau *et al*, 2002b; Hansen *et al*, 2003). Based on published data for the various fluoroquinolones showing maximum serum concentration and subsequent declining values over the duration of conventional dosing (Table 3.3), an estimation of the time (T) the serum drug concentration remains above the MIC₉₀ and MPC₉₀ for the various quinolones against MSSA strains resulted in a rank order of potency as follows: moxifloxacin T > MIC₉₀ > 24 hr and T > MPC₉₀ > 24 hr > levofloxacin (>24 hr and ~18 hr respectively) > gatifloxacin (>24 hr and ~12 hr respectively) > gemifloxacin (~24 hr and ~9 hr respectively).

It is argued that protein binding may affect drug concentrations required to eradicate an infection (Dalhoff and Schmitz, 2003). The percentage of protein binding for each fluoroquinolone is indicated in Table 3.3. An agent may show excellent *in vitro* data against an infecting organism, however, if the agent binds readily to proteins within the body the result may be less free (active) drug available for destroying the bacteria. This may lead to higher concentrations necessary to eradicate an infection. As such, pharmacokinetic parameters such as area under the concentration curve (AUC)/MIC and C_{max}/MIC may be calculated with regards to the amount of free drug available. These parameters are believed to be important in predicting the therapeutic outcome of patients with bacterial infections. The impact of protein binding on drug concentrations necessary for eradication of bacterial infections, however, is still under investigation and there is no proof to date of protein binding significantly affecting the concentrations needed for bacterial eradication.

3.2 MIC Results of *S. aureus* Isolates for Several Antimicrobial Agents

Twenty-six of the 122 MSSA isolates were selected for further susceptibility studies with five non-quinolone agents. The 22 MRSA were also further studied with the same non-quinolone agents. Two additional MRSA isolates were collected and tested against the same antimicrobials. MIC values for the 26 MSSA and 24 MRSA

were determined against nine antimicrobial agents. For comparison reasons, the fluoroquinolone MIC data presented above will once again be described in this section. The distribution of MIC values for MSSA and MRSA are shown in Table 3.4. Based on the MIC₉₀, the most potent antimicrobial for MSSA was gemifloxacin (0.016 µg/ml) followed by moxifloxacin (0.063 µg/ml) > garenoxacin (0.25 µg/ml) = levofloxacin (0.25 µg/ml) = cloxacillin (0.25 µg/ml) > gatifloxacin (1 µg/ml) = vancomycin (1 µg/ml) > cefazolin (2 µg/ml) > azithromycin (16 µg/ml). The most potent antimicrobial for MRSA, based on MIC₉₀ values, was vancomycin (1 µg/ml) followed by garenoxacin (2 µg/ml) > moxifloxacin (4 µg/ml) > gatifloxacin (8 µg/ml) = gemifloxacin (8 µg/ml) > cefazolin (16 µg/ml) > cloxacillin (32 µg/ml) = levofloxacin (32 µg/ml) > azithromycin (>512 µg/ml). The *in vitro* potency based on MIC values is currently used for determining the dosing strategies for achieving and maintaining targeted drug concentrations for different antimicrobials against *S. aureus*.

3.3 MPC Results of *S. aureus* Isolates for Several Antimicrobial Agents

For the above isolates in which MIC values were determined, MPC values were also determined with the exception of 6 MSSA isolates against azithromycin, 17 MRSA against azithromycin, and 1 MSSA isolate against gatifloxacin. MPC values were unavailable for these isolates for varying reasons. For 20 isolates missing MPC data against azithromycin, the MPC experiment was not performed because the MIC values were extremely high (>512 µg/ml). As MPC applies to organisms deemed to be susceptible to the antimicrobial agent by standardized susceptibility testing, MPC values would likely be inappropriate in such cases where the MIC value is above the NCCLS breakpoint (NCCLS, 2001a,b). Such high concentrations would not be clinically achievable in the serum, therefore, MPC evaluations were not performed on these isolates. The other four isolates missing MPC data were for reasons based on purity of the culture, obtaining the correct inoculum, or the availability of the agent. The fluoroquinolone MPC₉₀ data presented in section 3.1 will once again be described as a comparison to the MPC₉₀ data observed with the non-quinolone agents. The number of isolates tested by both MIC and MPC was 50 for cefazolin, cloxacillin, garenoxacin, gemifloxacin, levofloxacin, moxifloxacin, and vancomycin. For

Table 3.4: Distribution of minimum inhibitory concentration (MIC) values of *S. aureus* isolates (µg/ml).

Antimicrobial Agent	Susceptibility	n ¹	≤0.004	≤0.008	0.008	0.016	0.031	≤0.063	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	>512
Azithromycin	MSSA ²	26										1	13	6		2	1			3
	MRSA ³	24											5	2						17
	Total	50	0	0	0	0	0	0	0	0	0	1	18	8	0	2	1	0	0	20
Cefazolin	MSSA	26								2	4	15	1	3	1					
	MRSA	24											3	3	1	8	9			
	Total	50	0	0	0	0	0	0	0	2	4	15	4	6	2	8	9	0	0	0
Cloxacillin	MSSA	26						7		9	8	2								
	MRSA	24								4	10	1	2	4				2	1	
	Total	50	0	0	0	0	0	7	0	13	18	3	2	4	0	0	0	2	1	0
Garenoxacin	MSSA	26			4	11	4		2	1	1	2	1							
	MRSA	24				6	3					1	2	12						
	Total	50	0	0	4	17	7	0	2	1	1	3	3	12	0	0	0	0	0	0
Gatifloxacin	MSSA	26					9		5	4	4		1	3						
	MRSA	24					1		7		2				7	7				
	Total	50	0	0	0	0	10	0	12	4	6	0	1	3	7	7	0	0	0	0
Gemifloxacin	MSSA	26		20		4					1	1								
	MRSA	24		8		2								6	5	1	2			
	Total	50	0	28	0	6	0	0	0	0	1	1	0	6	5	1	2	0	0	0
Levofloxacin	MSSA	26							4	13	7					2				
	MRSA	24							1	7	1	1				8	3	2	1	
	Total	50	0	0	0	0	0	0	5	20	8	1	0	0	0	10	3	2	1	0
Moxifloxacin	MSSA	26	2		3	9	7		3				2							
	MRSA	24				7	2			1	2		2	7	2	1				
	Total	50	2	0	3	16	9	0	3	1	2	0	4	7	2	1	0	0	0	0
Vancomycin	MSSA	26										20	5		1					
	MRSA	24										19	4	1						
	Total	50	0	0	0	0	0	0	0	0	0	39	9	1	1	0	0	0	0	0

¹n=number of isolates tested

²MSSA=Methicillin-Susceptible *S. aureus*

³MRSA=Methicillin-Resistant *S. aureus*

azithromycin, 27 isolates were tested by both MIC and MPC and for gatifloxacin, 49 isolates were tested by both MIC and MPC. The results are shown in Table 3.5.

For MSSA infections, according to the MPC₉₀ values, the most potent agent out of the nine agents tested was gemifloxacin (0.125 µg/ml). After gemifloxacin, from most to least potent were moxifloxacin (0.5 µg/ml) > levofloxacin (1 µg/ml) > gatifloxacin (2 µg/ml) = garenoxacin (2 µg/ml) = cloxacillin (2 µg/ml) > vancomycin (4 µg/ml) > azithromycin (32 µg/ml) > cefazolin (64 µg/ml). For MRSA isolates, the most potent antimicrobial agents were vancomycin (8 µg/ml) and garenoxacin (8 µg/ml). After vancomycin and garenoxacin, from most to least potent for MRSA infections were moxifloxacin (16 µg/ml) > azithromycin (32 µg/ml) = gatifloxacin (32 µg/ml) > levofloxacin (128 µg/ml) > gemifloxacin (256 µg/ml) > cefazolin (512 µg/ml) > cloxacillin (>512 µg/ml). For MRSA, such high MPC values to cloxacillin were expected, since cloxacillin is closely related to methicillin. The MPC values may be important to address when determining new dosing strategies aimed at preventing resistance.

3.4 Comparison of the MIC and MPC Results

The MIC and MPC data of *S. aureus* isolates against nine different antimicrobials are shown in Table 3.6. For MSSA and MRSA strains tested against azithromycin (Table 3.6), the MIC₉₀ was equal to 2 µg/ml and the MPC₉₀ for these isolates was 32 µg/ml. The MPC range was 8->512 µg/ml. The MPC₉₀ was 16-fold higher than the MIC₉₀ for MSSA and MRSA against azithromycin. No significant difference between MSSA and MRSA was observed for these organisms tested against azithromycin. The MPC values appeared to be consistently 16-fold above the MIC values for both MSSA and MRSA strains.

For clinical isolates tested against cefazolin, MSSA and MRSA results differed greatly (Table 3.6). For MSSA isolates, the MIC₉₀ was 2 µg/ml. The MPC mode was 1 µg/ml while the MPC₉₀ was 64 µg/ml. For MRSA isolates, the MIC mode and the MIC₉₀ were 16 µg/ml. The MPC mode and MPC₅₀ were 256 µg/ml. The MPC₉₀ was 512 µg/ml. For MSSA and MRSA, the MPC₉₀ was 32-fold above the MIC₉₀. This shows that for both MSSA and MRSA isolates, the MPC values were consistently

Table 3.5: Distribution of mutant-prevention concentration (MPC) values of *S. aureus* isolates (µg/ml).

Antimicrobial Agent	Methicillin Susceptibility	n ¹	0.031	0.063	0.125	0.25	0.5	1	>1	2	4	>4	8	16	32	64	128	256	512	>512
Azithromycin	MSSA ²	20											5	4	9				1	1
	MRSA ³	7											3	1	2					1
	Total	27	0	0	0	0	0	0	0	0	0	0	8	5	11	0	0	0	1	2
Cefazolin	MSSA	26					1	9		1	1			4	6	1	1	2		
	MRSA	24															5	16	3	
	Total	50	0	0	0	0	1	9	0	1	1	0	0	4	6	1	6	18	3	0
Cloxacillin	MSSA	26					11	10		3	1								1	
	MRSA	24																1	1	22
	Total	50	0	0	0	0	11	10	0	3	1	0	0	0	0	0	0	1	2	22
Garenoxacin	MSSA	26		15	6		1			2	1		1							
	MRSA	24		6	2	1					1		13	1						
	Total	50	0	21	8	1	1	0	0	2	2	0	14	1	0	0	0	0	0	0
Gatifloxacin	MSSA	25				22				1	1		1							
	MRSA	24				10								9	4	1				
	Total	49	0	0	0	32	0	0	0	1	1	0	1	9	4	1	0	0	0	0
Gemifloxacin	MSSA	26		5	19					1	1									
	MRSA	24		3	7									6	5			1	2	
	Total	50	0	8	26	0	0	0	0	1	1	0	0	6	5	0	0	1	2	0
Levofloxacin	MSSA	26					21	2				1		1	1					
	MRSA	24					7	1				1	1			11	3			
	Total	50	0	0	0	0	28	3	0	0	0	2	1	1	1	11	3	0	0	0
Moxifloxacin	MSSA	26			17	5	1	1			2									
	MRSA	24			5	3		1	1				11	3						
	Total	50	0	0	22	8	1	2	1	0	2	0	11	3	0	0	0	0	0	0
Vancomycin	MSSA	26								2	23			1						
	MRSA	24									11		11	2						
	Total	50	0	0	0	0	0	0	0	2	34	0	11	3	0	0	0	0	0	0

¹n=number of isolates tested ²MSSA=Methicillin-Susceptible *S. aureus* ³MRSA=Methicillin-Resistant *S. aureus*

Table 3.6 Comparison of the minimum inhibitory concentration (MIC) and the mutant-prevention concentration (MPC) data for *S. aureus* isolates (µg/ml).

Antimicrobial Agent	Susceptibility	n ¹	MIC mode	MIC ₅₀ ⁴	MIC ₉₀ ⁴	MIC Range	MPC mode	MPC ₅₀ ⁵	MPC ₉₀ ⁵	MPC Range
Azithromycin	MSSA ²	20	1	1	2	0.5-8	32	32	32	8->512
	MRSA ³	7	1	1	2	1.0-2.0	8	16	32	8->512
Cefazolin	MSSA	26	0.5	0.5	2	0.125-4	1	16	64	1-256
	MRSA	24	16	8	16	1.0-16	256	256	512	128-512
Cloxacillin	MSSA	26	0.125	0.125	0.25	<=0.063-0.5	0.5	1	2	0.5-512
	MRSA	24	0.25	0.25	32	0.125-64	>512	>512	>512	256->512
Garenoxacin	MSSA	26	0.016	0.016	0.25	0.008-1	0.063	0.063	2	0.063-8
	MRSA	24	2	1	2	0.016-2	8	8	8	0.063-16
Gatifloxacin	MSSA	25	0.031	0.063	1	0.031-2	0.25	0.25	2	0.25-8
	MRSA	24	0.063, 4, 8	4	8	0.031-8	0.25	16	32	0.25-64
Gemifloxacin	MSSA	26	<=0.008	<=0.008	0.016	<=0.008-0.5	0.125	0.125	0.125	0.063-4
	MRSA	24	<=0.008	2	8	<=0.008-16	0.125	16	256	0.063-512
Levofloxacin	MSSA	26	0.125	0.125	0.25	0.063-8	0.5	0.5	1	0.5-32
	MRSA	24	8	8	32	0.063-64	64	64	128	0.5-128
Moxifloxacin	MSSA	26	0.016	0.016	0.063	<=0.004-1	0.125	0.125	0.5	0.125-4
	MRSA	24	0.016, 2	0.25	4	0.016-8	8	8	16	0.125-16
Vancomycin	MSSA	26	0.5	0.5	1	0.5-4	4	4	4	2.0-16
	MRSA	24	0.5	0.5	1	0.5-2	4 and 8	8	8	4.0-16

¹n=Number of isolates tested

²MSSA=Methicillin-Susceptible *S. aureus*

³MRSA=Methicillin-Resistant *S. aureus*

⁴MIC₅₀, MIC₉₀=Drug concentrations at which 50% and 90% of the strains were inhibited, respectively

⁵MPC₅₀, MPC₉₀=Drug concentrations at which resistant sub-populations for 50% and 90% of the strains were inhibited, respectively

higher than the MIC values. Furthermore, the MPC values observed for MRSA isolates were higher than those observed for MSSA isolates.

For MSSA isolates tested against cloxacillin (Table 3.6) the MIC₉₀ was 0.25 µg/ml and the MPC₉₀ was 2 µg/ml. For MRSA against cloxacillin, the MIC mode and MIC₅₀ were 0.25 µg/ml, whereas, the MIC₉₀ was 32 µg/ml. According to the NCCLS, some MRSA isolates may show *in vitro* susceptibility to β-lactams (as seen by the MIC mode and MIC₅₀) however, are not effective clinically and as such, are not recommended for treatment of MRSA (NCCLS, 2001a). It is still interesting, however, to analyze the susceptibility behavior of MRSA with cloxacillin. The MPC mode, MPC₅₀, and MPC₉₀ were >512 µg/ml. For MSSA and MRSA respectively, the MPC₉₀ values were 8 and >16-fold above the respective MIC₉₀ values. Cloxacillin is closely related to methicillin, therefore, it is logical that the MRSA organisms would have a decreased susceptibility pattern compared to the MSSA isolates as observed with the MIC₉₀ and MPC₉₀ values.

Table 3.6 also shows that garenoxacin had an MIC mode and an MIC₅₀ of 0.016 µg/ml for MSSA while the MIC₉₀ was 0.25 µg/ml. The MPC mode and MPC₅₀ were 0.063 µg/ml, while the MPC₉₀ was at 2 µg/ml. For MRSA isolates, the MIC₉₀ and MIC range were 2 and 0.016-2 µg/ml, respectively. The MPC mode, MPC₅₀, and MPC₉₀ were 8 µg/ml and the range recorded was 0.063-16 µg/ml. For MSSA, the MPC₉₀ was 8-fold above the MIC₉₀. For MRSA, the MPC₉₀ was 4-fold above the MIC₉₀.

MSSA isolates tested against gatifloxacin (Table 3.6) had a MIC₉₀ of 1 µg/ml and a MPC₉₀ of 2 µg/ml. The MPC₉₀ was 2-fold higher than the MIC₉₀ for MSSA isolates tested against gatifloxacin. For MRSA isolates tested against gatifloxacin, the MIC mode occurred at 3 different drug concentrations (0.063, 4, and 8 µg/ml). Seven isolates had MIC values at each of the three concentrations. The MIC₉₀ was 8 µg/ml. The MPC mode and the MPC₉₀ were 0.25 and 32 µg/ml, respectively. The MPC₉₀ was 4-fold higher than the MIC₉₀.

For isolates that were tested against gemifloxacin (Table 3.6), MSSA results were ≤0.008 µg/ml for both the MIC mode and the MIC₅₀. The MIC₉₀ and MIC range were 0.016 and ≤0.008-0.5 µg/ml, respectively. For MSSA isolates against gemifloxacin, 0.125 µg/ml was the drug concentration of the MPC mode, MPC₅₀, and

MPC₉₀. The MPC₉₀ was 8-fold higher than the MIC₉₀ for MSSA isolates. For MRSA isolates tested against gemifloxacin, the MIC mode was ≤ 0.008 $\mu\text{g/ml}$. The MIC₉₀ was 8 $\mu\text{g/ml}$. The MPC mode, MPC₅₀, and MPC₉₀ were 0.125, 16, and 256, respectively. For MRSA isolates, the MPC₉₀ was 32-fold above the MIC₉₀.

The MIC₉₀ for MSSA isolates tested against levofloxacin (Table 3.6) was 0.25 $\mu\text{g/ml}$. The MPC₉₀ was 1 $\mu\text{g/ml}$ and the MPC range was 0.5-32 $\mu\text{g/ml}$. For MRSA, the MIC mode and MIC₅₀ were 8 $\mu\text{g/ml}$, whereas the MIC₉₀ was 32 $\mu\text{g/ml}$. The MPC₉₀ and the MPC range were 128 and 0.5-128 $\mu\text{g/ml}$, respectively. For both MSSA and MRSA, the MPC₉₀ value was 4-fold above the MIC₉₀ value.

For MSSA isolates tested against moxifloxacin (Table 3.6), the MIC₉₀ value was 0.063 $\mu\text{g/ml}$. The MPC₉₀ was 0.5 $\mu\text{g/ml}$. For MRSA isolates against moxifloxacin, the MIC mode was 0.016 and 2 $\mu\text{g/ml}$ with seven isolates existing at each of these two drug concentrations. The MIC₉₀ was 4 $\mu\text{g/ml}$ and the MPC₉₀ was 16 $\mu\text{g/ml}$. The MPC₉₀ value for MSSA was 8-fold greater than the MIC₉₀ value. The MPC₉₀ value for MRSA was 4-fold greater than the MIC₉₀.

Vancomycin data were similar for both MSSA and MRSA strains (Table 3.6). The MIC₉₀ value for MSSA and MRSA was 1 $\mu\text{g/ml}$. The MPC mode, MPC₅₀, and MPC₉₀ were all at 4 $\mu\text{g/ml}$ for MSSA isolates. The MPC₉₀ for MRSA was 8 $\mu\text{g/ml}$. The MPC₉₀ value for MSSA was 4-fold higher than the MIC₉₀ value. The MPC₉₀ value for MRSA was 8-fold higher than the MIC₉₀ value.

Individual colonies growing on plates containing elevated drug concentrations were collected (MPC-recovered isolates) and stored in 500 μl of skim milk at -70°C . MPC-recovered isolates have been described as recovered subpopulations with higher drug concentrations required for inhibition compared to the original heterogeneous population (parental population) from which the subpopulation was selected (Hansen *et al*, 2001). The MPC-recovered isolates were re-tested by MIC methodologies (Table 3.7). Except for vancomycin, all MPC-recovered isolates with high MPC values had elevated MIC levels compared to their original parental MIC values.

3.5 Overcrowding Results

The issue concerning overcrowding was evaluated using *S. aureus* isolate #4 and the drug levofloxacin. Overcrowding is the growth of an organism due to the lack of

Table 3.7: Minimum inhibitory concentrations (MICs) of recovered *S. aureus* isolates from mutant-prevention concentration (MPC) drug plates.

Antimicrobial Agent	Organism #	Original MIC	Recovered MIC	Ratio
Azithromycin	4	1	>128	>128
	9	1	32	32
	21	1	>128	>128
	25	1	32	32
	34	1	64	64
	35	1	>128	>128
	41	1	>128	>128
Cefazolin	14	1	64	64
	35	0.25	64	256
	47	1	64	64
Cloxacillin	11	0.125	>64	>512
	13	0.125	>64	>512
	16	0.125	32	256
	35	<=0.064	32	>512
	36	0.25	32	128
	37	2	32	16
	40	0.125	>64	>512
	43	0.125	>64	>512
	48	0.125	>64	>512
Garenoxacin	14	2	16	8
Gatifloxacin	5	0.031	0.25	8
	10	0.031	0.25	8
	11	0.031	0.25	8
	24	0.031	0.25	8
	26	0.031	2	64
	27	0.031	0.25	8
	29	0.031	0.5	16
	33	0.031	0.25	8
	34	0.031	0.125	4
	35	0.031	0.125	4
Gemifloxacin	5	<=0.008	0.125	>16
	6	<=0.008	0.125	>16
	7	<=0.008	0.125	>16
	8	<=0.008	0.063	>8
	9	<=0.008	0.125	>16
	10	<=0.008	0.063	>8
	15	<=0.008	0.125	>16
	17	<=0.008	0.125	>16
	23	<=0.008	0.125	>16
	25	<=0.008	0.125	>16
	29	<=0.008	0.125	>16
	30	<=0.008	0.125	>16
	31	<=0.008	0.125	>16
	32	<=0.008	0.125	>16
	33	<=0.008	>1	>128
	41	<=0.008	1	128
Levofloxacin	21	0.063	0.5	8
	34	0.125	8	64
	35	0.063	16	256
	38	0.125	8	64
	39	0.125	8	64
	48	0.125	8	64
Moxifloxacin	17	0.016	0.25	16
	20	<=0.004	0.125	>32
	22	0.008	0.125	16
	23	0.008	0.125	16
	24	<=0.004	0.125	>32
	32	0.008	0.25	32
	33	0.016	2	128
	34	0.031	2	64
	35	0.016	2	128
	38	0.016	2	128
	39	0.016	4	128
Vancomycin	26	0.5	0.25	0.5
	41	0.5	0.5	1
	46	1	0.5	0.5
	48	0.5	0.5	1
	49	0.5	0.5	1
	50	0.5	0.5	1

contact of that organism to the antimicrobial agent. This was a concern for the MPC concept, since this test is based on a large inoculum of bacteria ($\geq 10^9$ cfu/ml) and with this large inoculum, there may be a possibility that the antimicrobial agent was not in contact with the organism due to excess organism interfering with other organism/drug contact. In this case, growth observed at high drug concentrations are due to the overcrowding phenomenon.

The results of the overcrowding experiment are displayed in Table 3.8. There was no significant change in MPC values when plating out various volumes of inoculum. One doubling dilution change occurred between plating 300 and 400 μ l of inoculum. This was insignificant, since the volume plated for *S. aureus* organisms was 100 μ l. The MPC results appeared consistent despite the various volumes of inoculum added to the plates.

3.6 Characterization of MPC-recovered Populations

3.6.1 Vancomycin MPC-recovered Populations

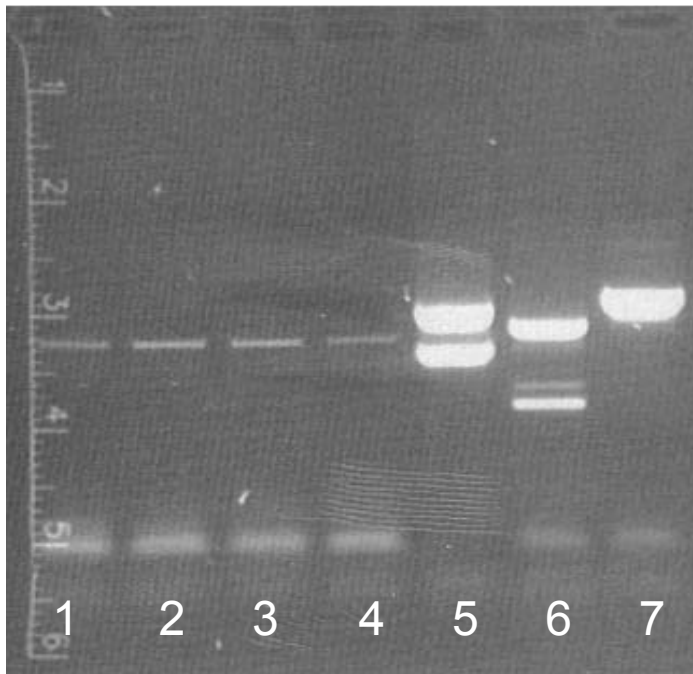
Some *S. aureus* isolates had elevated MPC values to vancomycin compared to the MIC values. When the MPC-recovered populations were re-tested for their MIC, elevations in the MIC value were not observed. Further characterization of the vancomycin MPC-recovered samples was performed to help determine the mechanism of resistance.

PCR was performed on the MPC-recovered populations that displayed high MPC values to vancomycin. Genes that confer glycopeptide resistance in enterococci include *vanA*, *vanB*, *vanC*, *vanC2/C3*, *vanD*, *vanE*, and *vanG* (McKessar *et al*, 2000). An attempt to detect these genes in the isolates with high MPC values proved unsuccessful (Figures 3.1-3.3). With the possibility of the isolates reverting back to susceptible phenotypes, samples were taken directly from their storage media and were prepared for PCR, as opposed to growing them overnight in the absence of vancomycin. Positive controls for *vanC2/C3*, *vanD*, *vanE*, and *vanG* were unavailable at the time of experimentation, however, recent studies using the primers listed in Table 2.1 proved that the primers were functioning as expected (data not shown). All tested samples were negative for all above vancomycin-resistant genes. Figure 3.1 shows organisms tested for the presence of *vanA*, *vanB*, and *vanC*. A faint band that looked to be the size

Table 3.8: Influence of inoculum volume on the mutant-prevention concentration (MPC) for levofloxacin.

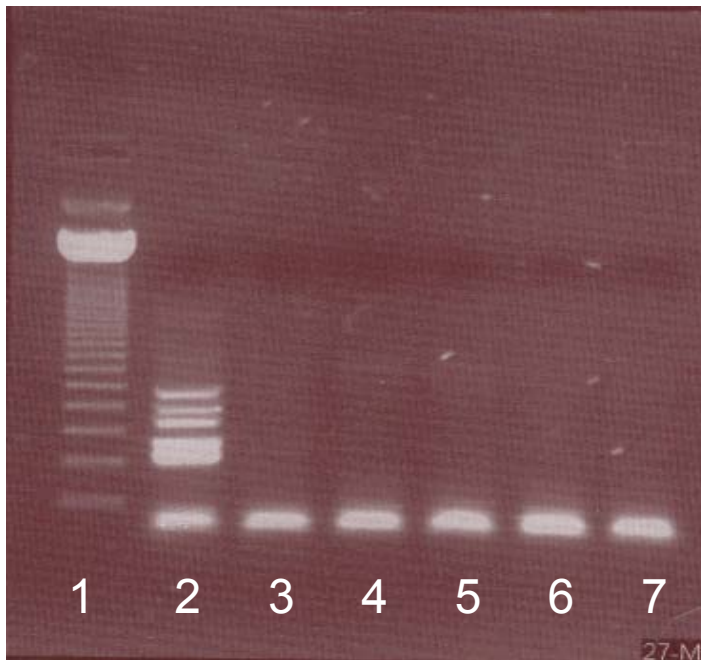
Volume added to the plates (µls)	cfu ¹ added to each plate	MPC (µg/ml)
25	1.3×10^8	0.5
50	2.6×10^8	0.5
100	5.1×10^8	0.5
150	7.7×10^8	0.5
200	1.0×10^9	0.5
250	1.3×10^9	0.5
300	1.5×10^9	0.5
400	2.1×10^9	1
600	3.1×10^9	1
800	4.1×10^9	1
1000	5.1×10^9	1
1250	6.4×10^9	1
1500	7.7×10^9	1
1750	9.0×10^9	1
2000	1.0×10^{10}	1

¹cfu=colony forming units



Lane 1=9-parent
 Lane 2=9-MPC-recovered
 Lane 3=15-parent
 Lane 4=15-MPC-recovered
 Lane 5=*vanA*-Positive Control
 Lane 6=*vanB*-Positive Control
 Lane 7=*vanC*-Positive Control

Figure 3.1: Polymerase chain reaction (PCR) for *vanA*, *vanB*, and *vanC* genes in *S. aureus* isolates with elevated MPC values (MPC-recovered).



Lane 1= 123 bp Marker
 Lane 2= *vanC*-Positive Control
 Lane 3= Negative Control
 Lane 4=41-MPC-recovered
 Lane 5=46-MPC-recovered
 (isolated from a plate
 containing 2 µg/ml
 vancomycin)
 Lane 6=46-MPC-recovered
 (isolated from a plate
 containing 4 µg/ml
 vancomycin)
 Lane 7=29213-MPC-recovered

Figure 3.2: Polymerase chain reaction (PCR) for *vanC2/C3* and *vanD* genes in *S. aureus* isolates with elevated MPC values (MPC-recovered).

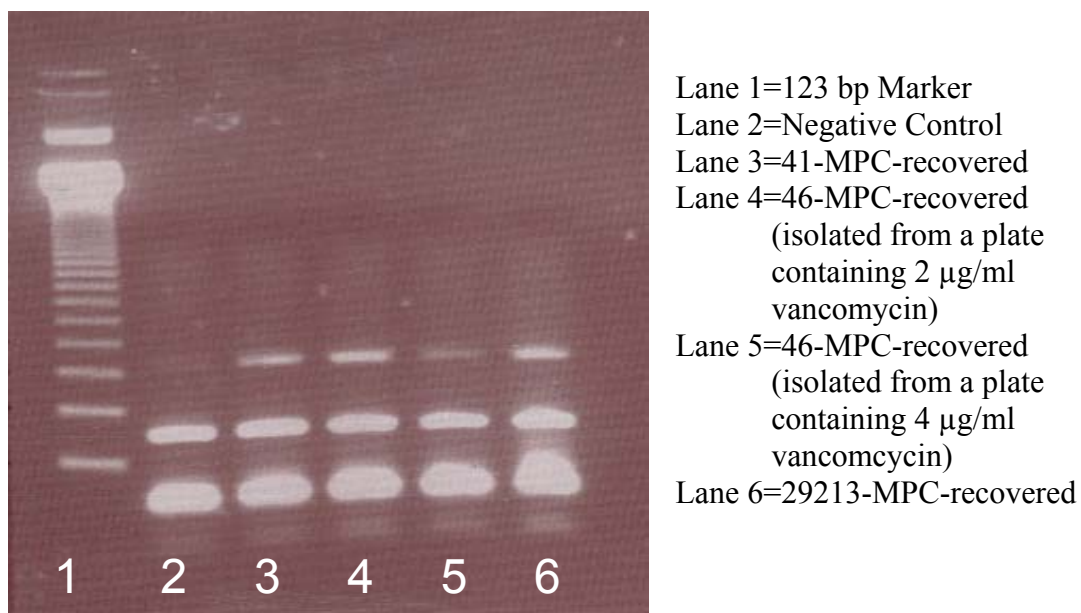


Figure 3.3: Polymerase chain reaction (PCR) for *vanE* and *vanG* genes in *S. aureus* isolates with elevated MPC values (MPC-recovered).

of *vanB* was observed, however, this band appeared in both the MPC-recovered and the parental isolates, therefore, it was not the cause of the increased MPC value observed in the MPC-recovered population. Figure 3.2 displays the gel for the *vanC2/C3* and *vanD* genes. Figure 3.3 shows non-specific binding occurring at approximately 200 and 400 bp. These bands do not correspond to the expected sizes of *vanE* (513 bp) and *vanG* (810 bp) and were, therefore, disregarded.

A MPC-recovered population that had an elevated MPC value for vancomycin (8 µg/ml) was further studied using Electron Microscopy. The cells were grown on media containing drug to prohibit the population from reverting back to a wild type susceptible phenotype. Cells were then fixed with glutaraldehyde and given to Karen Slattery (Electron Microscopy Technician, Department of Pathology, SHR) for slicing, staining, and analysis. Blind measurements of the cell walls of populations #41 and #41m were recorded. An electron micrograph of the susceptible parent population (#41) at 100,000X is shown in Figure 3.4. Figure 3.5 is the electron micrograph of the intermediate-resistant MPC-recovered population (#41m) at 100,000X.

Statistical analysis was performed on the cell wall measurements to determine if the difference observed in cell wall thickness was significant. The mean thickness of the cell wall from the susceptible parental population ($\mu_{\#41}$) was 327 ± 37 Å. The mean thickness of the cell wall from the MPC-recovered population ($\mu_{\#41m}$) was 466 ± 113 Å. At the 1% significance level ($\alpha=0.01$), the critical value (Z_c) was approximately 2.33 (Mann, 1998a). The test value (Z_t) was calculated to be 6.6. The test value ($Z_t=6.6$) exceeded the critical value ($Z_c=2.33$), therefore, the null hypothesis (H_0) which stated that the cell wall populations were equal at 327 Å was rejected. The mean thickness of the cell wall of population #41m (466 Å) was too far from the hypothesized population of $\mu_{\#41}=327$ Å to be due to chance or sampling error alone. The cell wall of population #41m was, indeed, significantly thicker than the cell wall of population #41.

3.6.2 Azithromycin MPC-recovered Populations

Isolates with high MPC values to the azalide, azithromycin, were tested for the presence of macrolide-resistant genes (*ermA*, *ermB*, *ermC*, *mefA/E*, and *msrA/B*) by PCR. The MPC-recovered populations of isolates 4 (Az4), 34 (Az34), and 35 (Az35) were positive for the *ermC* gene (Figure 3.6). The other isolates tested (Az9, Az25, and

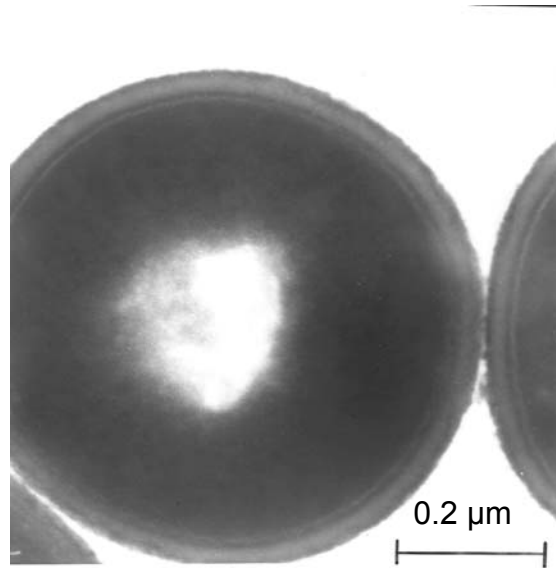


Figure 3.4: Electron microscopy (EM) of a susceptible parent population (#41).

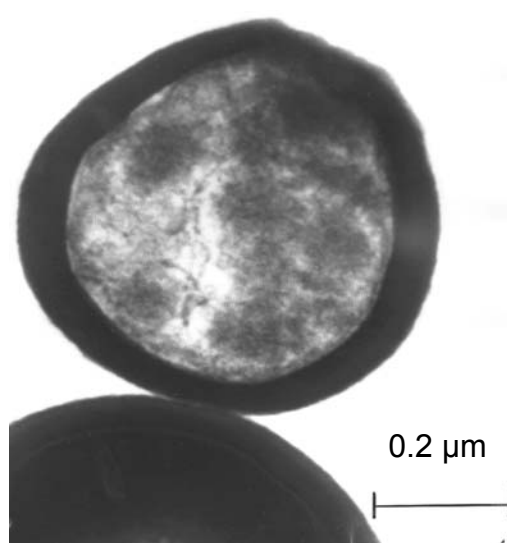


Figure 3.5: Electron microscopy (EM) of an intermediate-resistant MPC-recovered population (#41m).

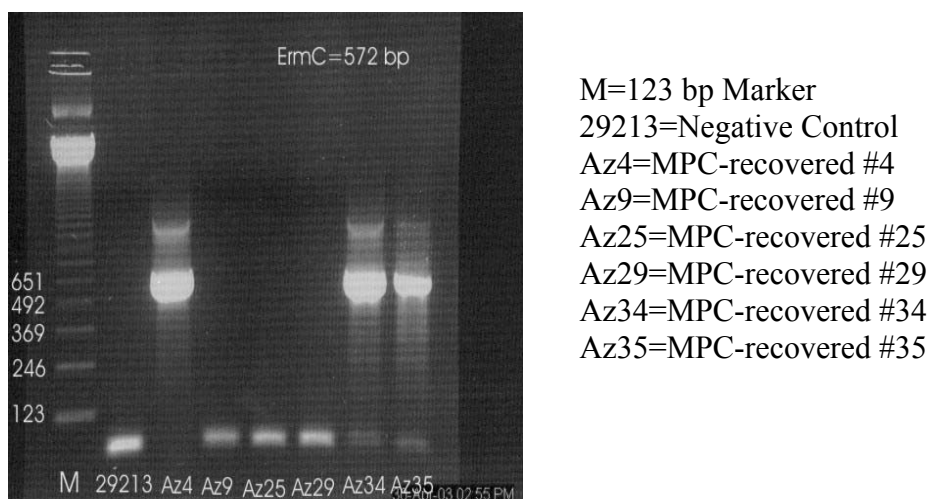


Figure 3.6: Polymerase chain reaction (PCR) for *ermC* in MPC-recovered populations from azithromycin drug plates.

Az29) were *ermC*-negative. No positive control was available, however, the presence of the *ermC* gene was confirmed through sequencing and NCBI (National Centre for Biotechnology Information) searches. The NCBI BLAST search revealed that the sample sequence had a 98% homology to the *ermC* gene in *S. aureus* M798 (gi | 18542235). Four base pairs out of 346 base pairs did not match up. This may be because the strain used in this study was a clinical *S. aureus* strain and was likely different from the strain in the database. Also, the search indicated that there was a 0% chance that the homology between the query and the *S. aureus ermC* gene was due to randomness (E-value=0). The parents of these MPC-recovered populations (4, 34, and 35) were all negative for the *ermC* gene (Figure 3.7).

S. pneumoniae MIC and MPC data were collected (Table 3.9). I further characterized the isolates with elevated MPC values by PCR. Like the *S. aureus* MPC-recovered populations that were *ermC*-positive, some MPC-recovered populations of *S. pneumoniae* had macrolide-resistant genes as well (Table 3.9). Figure 3.8 shows an example of both *mefA*- and *ermB*-positive MPC-recovered populations. The parents of these MPC-recovered populations were negative for the macrolide-resistant genes. An example gel is shown in Figure 3.9. Isolates A2, A6, A7, and A12 had acquired the macrolide efflux gene, *mefA*. Isolates A5 and A15 had acquired the erythromycin resistance methylase gene, *ermB* (Blondeau *et al*, 2004).

3.7 Application of the MIC and MPC Results

3.7.1 Pharmacokinetic Curves

According to Zhao and Drlica (2001), the mutant-selection window (MSW) is an important clinical parameter that exists between the MIC and the MPC values. At an inoculum of $\geq 10^9$ cfu/ml, the MSW signifies the concentrations of drug that optimally select resistant phenotypes. Serum concentrations within the MSW select resistant subpopulations as discussed in section 1.4.4.1. It is desirable to minimize the time period that the serum drug concentration is within the MSW to prevent resistant subpopulations from proliferating. An optimal drug to use would be one with a narrow MSW thereby decreasing the risk of emerging resistant mutants. Another possibility for preventing resistance is by treating at or above the MPC value thereby moving drug concentrations away from the MSW. Hence, the MSW may be a significant factor to

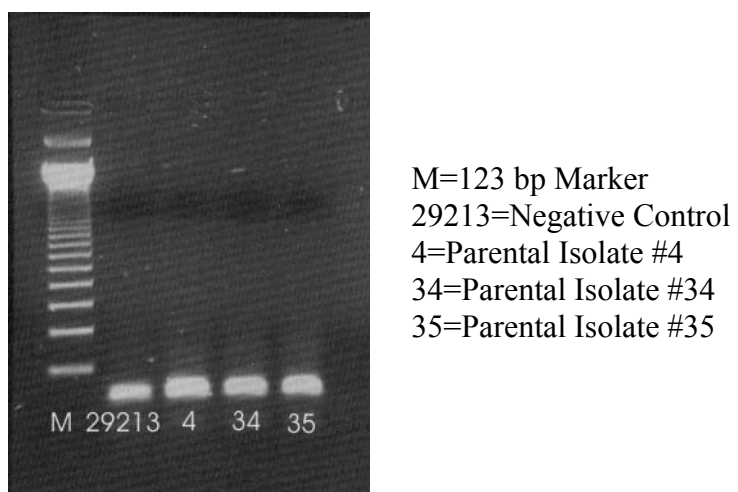


Figure 3.7: Polymerase chain reaction (PCR) for *ermC* in parental isolates of MPC-recovered populations of *S. aureus* that were positive for *ermC*.

Table 3.9: *S. pneumoniae* isolates with elevated mutant-prevention concentration (MPC) (µg/ml) levels and the presence or absence of macrolide-resistance determinants.

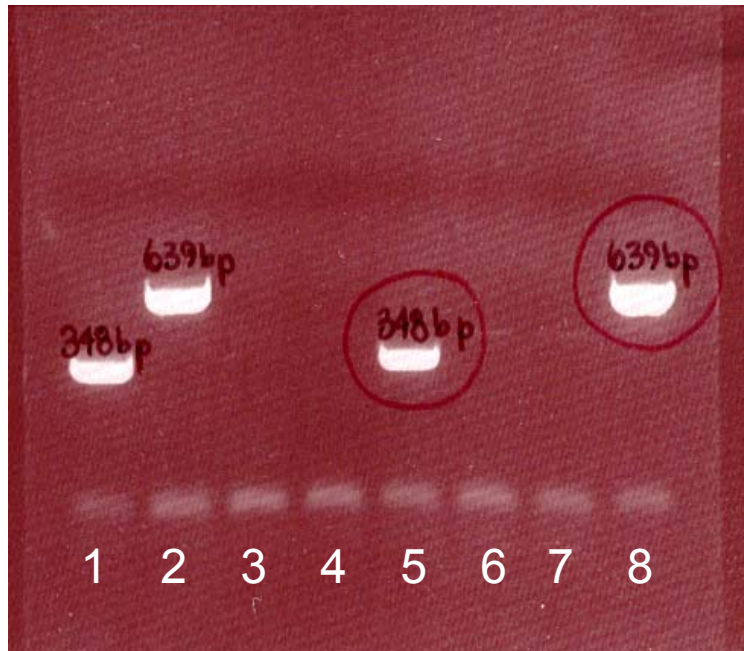
Isolate #	Selecting Agent	Penicillin MIC ¹	Penicillin Susceptibility	MIC ¹	MPC	MIC _{rec} ²	<i>mefA</i> ³	<i>ermB</i> ⁴
A1	Erythromycin	<=0.008	Sensitive	0.063	0.5	4	-	-
A2	Erythromycin	<=0.008	Sensitive	0.063	>=4	8	+	-
A3	Erythromycin	0.016	Sensitive	0.031	2	16	-	-
A4	Erythromycin	0.125	Intermediate	0.125	>=4	>16	-	-
A5	Erythromycin	0.016	Sensitive	0.031	>=4	>16	-	+
A6	Erythromycin	<=0.008	Sensitive	0.063	2	8	+	-
A7	Clarithromycin	<=0.008	Sensitive	0.063	1	4	+	-
A8	Clarithromycin	0.016	Sensitive	0.031	1	4	-	-
A9	Clarithromycin	<=0.008	Sensitive	0.031	1	1	-	-
A10	Azithromycin	0.016	Sensitive	0.25	>=8	>32	-	-
A11	Azithromycin	<=0.008	Sensitive	0.125	>=8	16	-	-
A12	Azithromycin	<=0.008	Sensitive	0.125	>=8	8	+	-
A13	Azithromycin	0.016	Sensitive	0.125	>=8	>32	-	-
A14	Azithromycin	<=0.008	Sensitive	0.063	2	8	-	-
A15	Azithromycin	0.016	Sensitive	0.063	>=8	>32	-	+
A16	Azithromycin	<=0.008	Sensitive	0.125	8	>32	-	-

¹MIC=Minimum Inhibitory Concentration (µg/ml)

²MIC_{rec}=Minimum Inhibitory Concentration (µg/ml) of isolates recovered from MPC plates

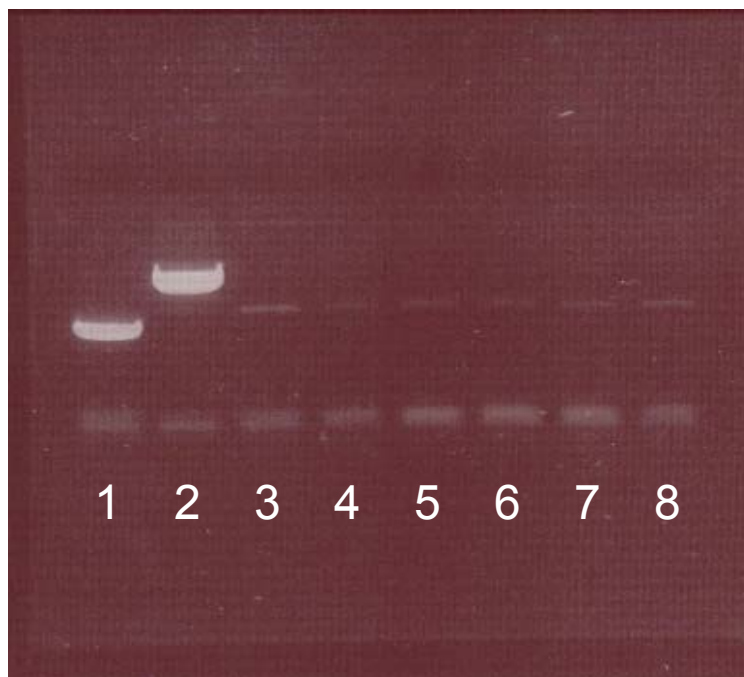
³*mefA*=Macrolide Efflux Gene

⁴*ermB*=Erythromycin Resistance Methylase Gene



Lane 1=*mefA*-Positive Control
 Lane 2=*ermB*-Positive Control
 Lane 3=Negative Control
 Lane 4=MPC-recovered A1
 Lane 5=MPC-recovered A2
 Lane 6=MPC-recovered A3
 Lane 7=MPC-recovered A4
 Lane 8=MPC-recovered A5

Figure 3.8: Polymerase chain reaction (PCR) for *mefA* and *ermB* in *S. pneumoniae* MPC-recovered populations from MPC plates containing erythromycin.



Lane 1=*mefA*-Positive Control
 Lane 2=*ermB*-Positive Control
 Lane 3=Negative Control
 Lane 4=MPC-recovered A16
 Lane 5=Parental Isolate A2
 Lane 6=Parental Isolate A5
 Lane 7=Parental Isolate A6
 Lane 8=Parental Isolate A7

Figure 3.9: Polymerase chain reaction (PCR) for *mefA* and *ermB* in *S. pneumoniae* parental isolates of the MPC-recovered populations showing the presence of either *mefA* or *ermB* genes.

consider when developing new dosing strategies for novel antimicrobials. All of the pharmacokinetic curves shown in this thesis were collected from various sources as indicated in each figure. The MIC₉₀, MPC₅₀, and MPC₉₀ values presented in this thesis were superimposed on each curve. Determination of the MSW was then possible for each antimicrobial agent.

3.7.1.1 Quinolones

3.7.1.1.1 Garenoxacin

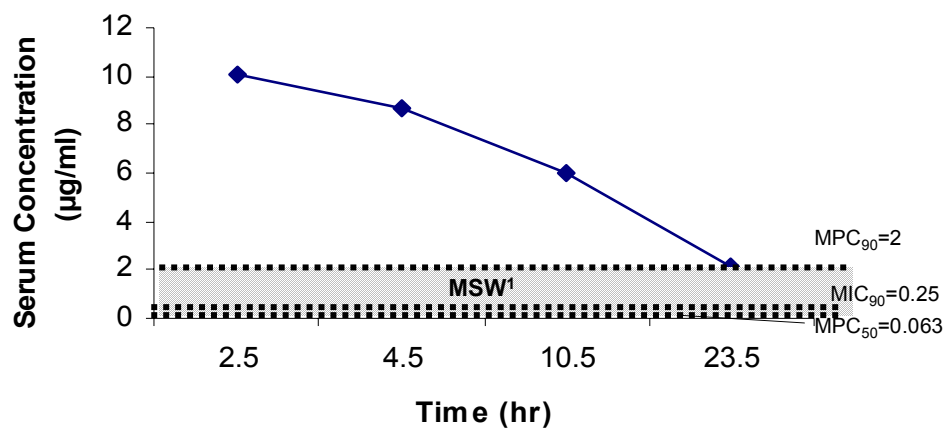
The MSW of MSSA to garenoxacin was 0.25-2 µg/ml based on a single 600 mg oral dose (Figure 3.10). At this dose, drug concentrations would remain above the MSW for the entire dosing period. Since the MPC₉₀ (2 µg/ml) was below the maximum serum concentration (~10 µg/ml), this level is achievable in patients, thereby preventing the selection of resistant subpopulations in a large heterogeneous population of cells.

The MSW of garenoxacin for MRSA isolates involved most of the pharmacokinetic curve at concentrations between 2 and 8 µg/ml (Figure 3.11). At the dosage recommended by the manufacturer, serum concentrations would lie within the MSW for ~17 hr. Time above the MPC₉₀ was 4.5-5.5 hr. Because the MPC₉₀ value existed below the maximum serum concentration, treatment at the MPC value may be achievable with minimal side effects.

3.7.1.1.2 Gatifloxacin

Gatifloxacin tested against MSSA showed that for the recommended dosage of 400 mg once a day, serum drug concentrations were within the MSW for ~6 hr (Figure 3.12). The MSW was 1-2 µg/ml. Time above the MPC₉₀ was ~6 hr. Treating above the MPC level may be achievable, since the MPC was 2 µg/ml and the maximum serum concentration was above 4 µg/ml.

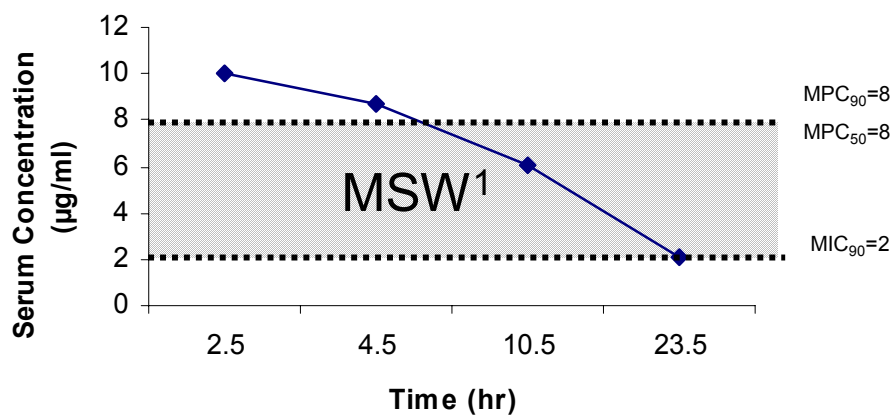
The gatifloxacin curve against MRSA portrayed a MSW (8-32 µg/ml) that existed above the maximum serum concentration (Figure 3.13). The MPC value is not achievable in the serum for this reason. According to the data presented, gatifloxacin serum concentrations do not exceed the MIC value, therefore, susceptible cells may continue to proliferate, especially in an immunocompromised individual (Tillotson *et al*, 2001).



Andrews *et al*, 2003

1MSW =Mutant-Selection Window

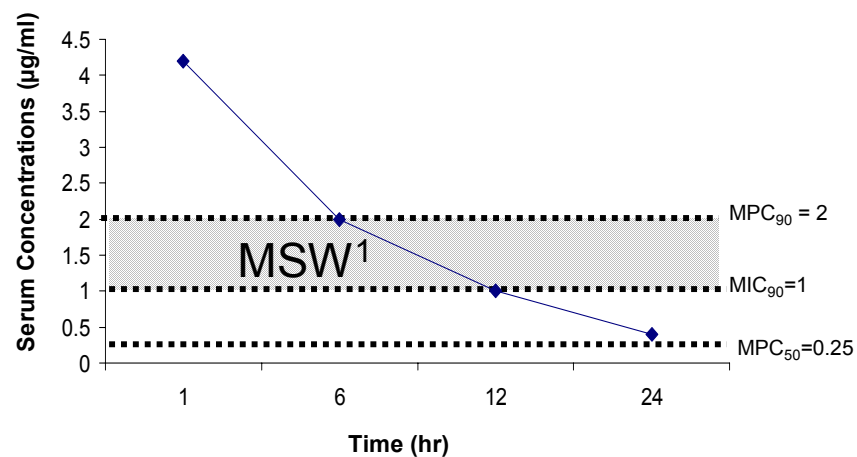
Figure 3.10: Relationship of serum concentration of garenoxacin to the mutant-prevention concentration (µg/ml) for methicillin-susceptible *S. aureus*.



Andrews *et al*, 2003

1MSW =Mutant-Selection Window

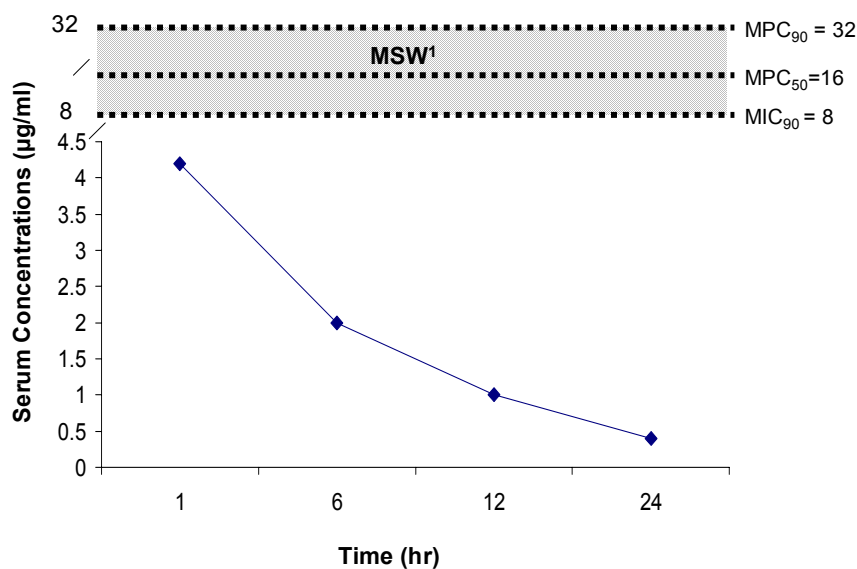
Figure 3.11: Relationship of serum concentration of garenoxacin to the mutant-prevention concentration (µg/ml) for methicillin-resistant *S. aureus*.



Blondeau *et al*, 2003b

¹MSW=Mutant-Selection Window

Figure 3.12: Relationship of serum concentration of gatifloxacin to the mutant-prevention concentration (µg/ml) for methicillin-susceptible *S. aureus*.



Blondeau *et al*, 2003b

¹MSW=Mutant-Selection Window

Figure 3.13: Relationship of serum concentration of gatifloxacin to the mutant-prevention concentration (µg/ml) for methicillin-resistant *S. aureus*.

3.7.1.1.3 Gemifloxacin

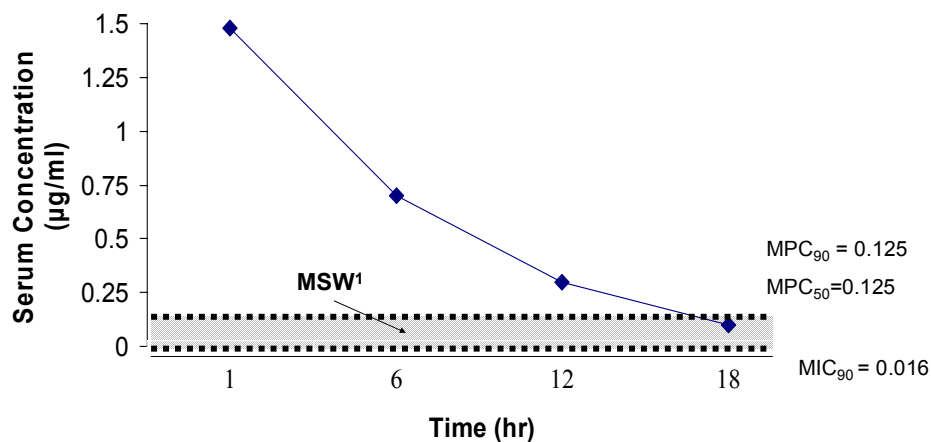
The MSW of gemifloxacin to MSSA strains appeared to be quite narrow and low on the pharmacokinetic curve (0.016-0.125 µg/ml) (Figure 3.14). There was, however, a small time frame of ~1.5 hr in which the concentration of drug was within the MSW at the dosage recommended by the manufacturers (320 mg once-daily). Re-administration of this agent before serum levels reach the MSW would move the serum concentrations away from the MSW. Because the MPC₉₀ (0.125 µg/ml) was lower than the maximum serum concentration (1.5 µg/ml), MPC₉₀ levels are achievable and sustainable within the serum of an infected patient. Based on the recommended dosage, time above the MPC₉₀ was ~16 hr.

For MRSA, the MSW was above the maximum serum concentration based on the MPC concept/model (the MSW was 8-256 µg/ml and the maximum serum concentration was ~1.5 µg/ml) (Figure 3.15). A similar observation was made with gatifloxacin and MRSA organisms. Treatment failure would likely be due to the outgrowth of susceptible cells, since the serum concentration does not reach even the MIC level (Tillotson *et al*, 2001).

3.7.1.1.4 Levofloxacin

The MSW for levofloxacin was low and narrow for MSSA organisms (0.25-1 µg/ml). At the end of the dosing period, however, there was ~6 hr in which the serum drug concentration existed within the MSW (Figure 3.16). Like gemifloxacin and MSSA, this may be avoided by re-administration of the drug before the serum concentration reaches the MSW (i.e., changing the dosage regimen). If this is practical, serum concentrations would cease to exist within the MSW, making this agent effective at resolving an infection and preventing the outgrowth of resistant subpopulations with minimal adverse reactions. For levofloxacin and MSSA, the serum drug concentrations remain above the MPC₉₀ for ~18 hr.

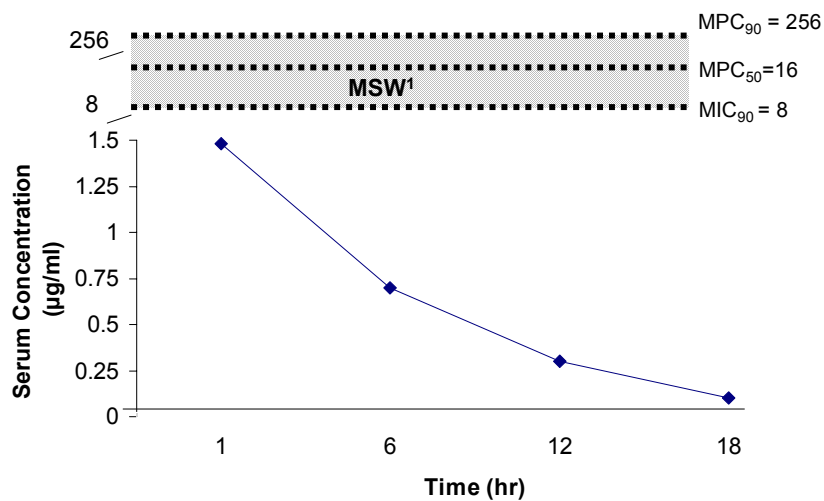
For MRSA isolates, the MSW existed above the maximum serum concentration (Figure 3.17). The maximum serum concentration was ~6 µg/ml and the MSW was 32-128 µg/ml. This was similar to the findings of gemifloxacin and gatifloxacin and MRSA isolates.



Glaxo-Smith Kline
Blondeau *et al*, 2003b

¹MSW=Mutant-Selection Window

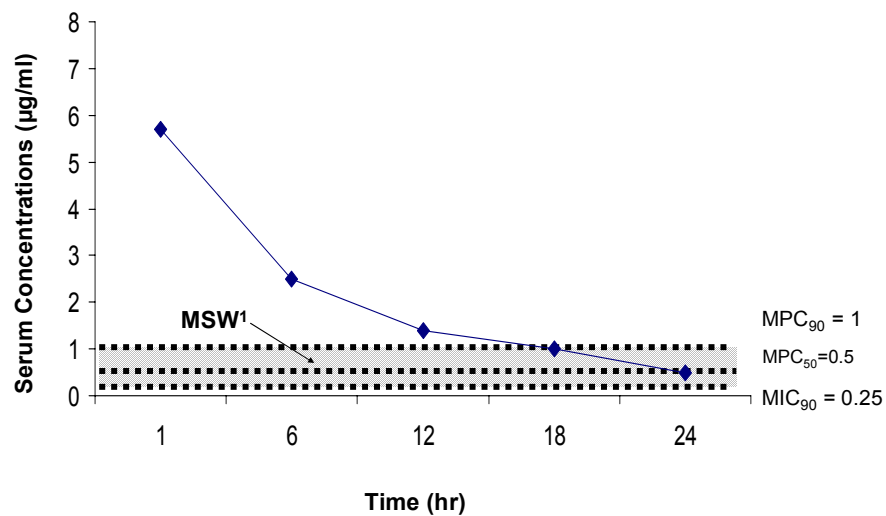
Figure 3.14: Relationship of serum concentration of gemifloxacin to the mutant-prevention concentration (µg/ml) for methicillin-susceptible *S. aureus*.



Glaxo-Smith Kline
Blondeau *et al*, 2003b

¹MSW=Mutant-Selection Window

Figure 3.15: Relationship of serum concentration of gemifloxacin to the mutant-prevention concentration (µg/ml) for methicillin-resistant *S. aureus*.

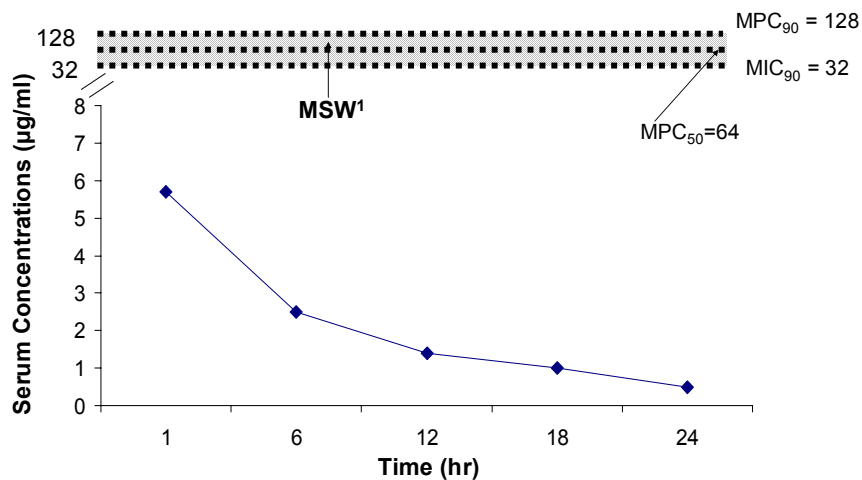


Blondeau *et al*, 2003b

¹MSW=Mutant-Selection Window

Janssen-Ortho

Figure 3.16: Relationship of serum concentration of levofloxacin to the mutant-prevention concentration (µg/ml) for methicillin-susceptible *S. aureus*.



Blondeau *et al*, 2003b

¹MSW=Mutant-Selection Window

Janssen-Ortho

Figure 3.17: Relationship of serum concentration of levofloxacin to the mutant-prevention concentration (µg/ml) for methicillin-resistant *S. aureus*.

3.7.1.1.5 Moxifloxacin

For moxifloxacin and MSSA, the MSW was below the pharmacokinetic curve and was very narrow (0.063-0.5 µg/ml) (Figure 3.18). The amount of time that the serum drug concentrations were within the MSW was zero (based on approved dosing), thereby decreasing the chance that a resistant phenotype to moxifloxacin may be selected out. Resistant phenotypes that may be present in large heterogeneous populations of cells would likely be destroyed along with the susceptible phenotypes by treatment with moxifloxacin at a 400 mg once-daily dosing.

For MRSA infections, the MSW was extremely high on the pharmacokinetic curve (4-16 µg/ml) and it involved the upper portion of the pharmacokinetic curve (Figure 3.19). Treatment failure may be the result of outgrowth of susceptible cells, since the serum drug concentration exceeded the MIC value for only a short period of time (~3 hr during the dosing period) (Tillotson *et al*, 2001). The MPC₉₀ value is likely unachievable, since the maximum serum concentration (~4.5 µg/ml) is below the MPC₉₀ value (16 µg/ml).

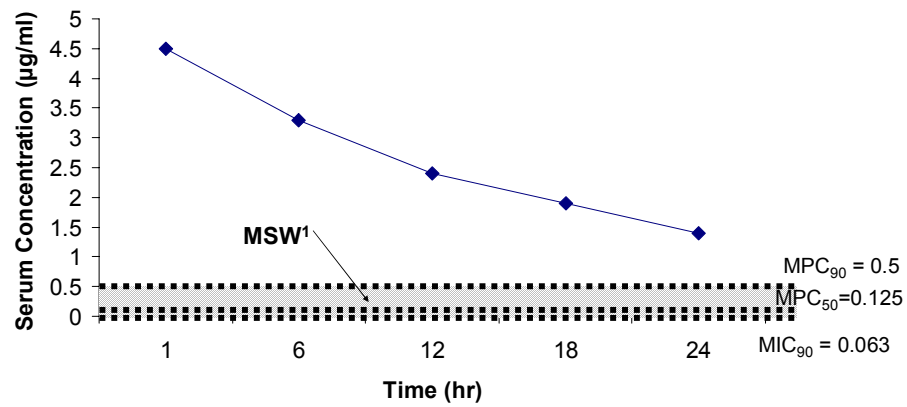
3.7.1.2 Glycopeptide

3.7.1.2.1 Vancomycin

The MSW for vancomycin against MSSA (Figure 3.20) included only a small portion of the pharmacokinetic curve after a dosage of 500 mg (Healy *et al*, 1987). The MSW for MSSA was 1-4 µg/ml. Re-administration of this agent before serum concentrations reach the MSW would move the serum levels away from the MSW.

The MSW for MRSA against vancomycin was 1-8 µg/ml (Figure 3.21). For MRSA systemic infections, the pharmacokinetic curve existed within the MSW for ~7 hr. Within this time, resistant subpopulations may be selected out of large heterogeneous populations. The MPC₉₀ levels may possibly be achievable in a patient, since these levels were below the maximum serum concentration which was ~40 µg/ml. Time spent above the MPC₉₀ value was ~7 hr.

Despite the overall observations showing the success of vancomycin activity, a small number of organisms resulted in MPC values that exceeded the NCCLS breakpoint for susceptible isolates (NCCLS, 2001a). These isolates were further studied by PCR and EM (section 3.6.1).

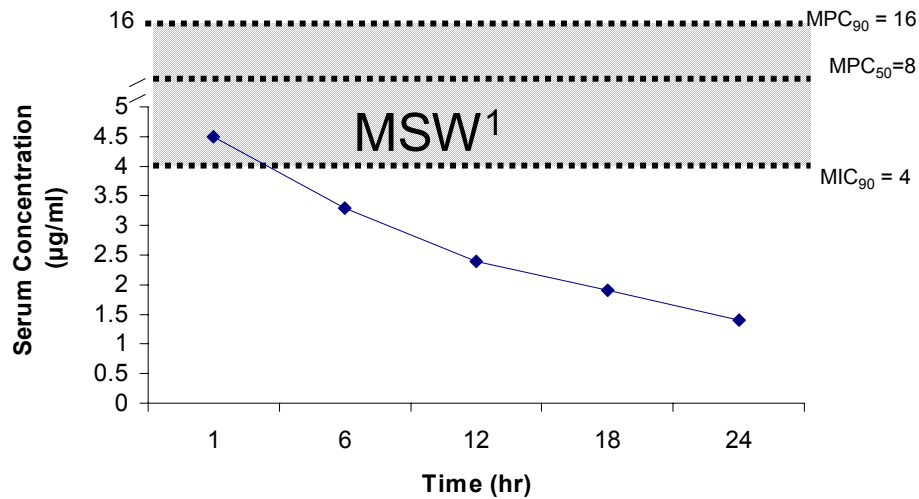


Wise *et al*, 1999

¹MSW=Mutant-Selection Window

Blondeau *et al*, 2000b

Figure 3.18: Relationship of serum concentration of moxifloxacin to the mutant-prevention concentration (µg/ml) for methicillin-susceptible *S. aureus*.

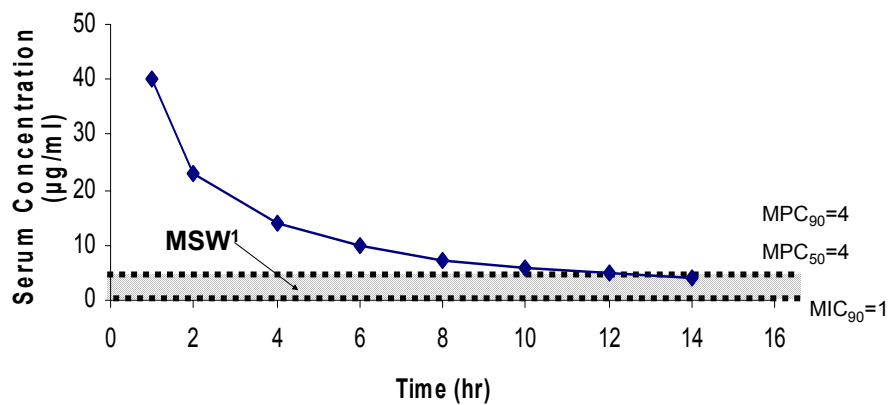


Wise *et al*, 1999

¹MSW=Mutant-Selection Window

Blondeau *et al*, 2003b

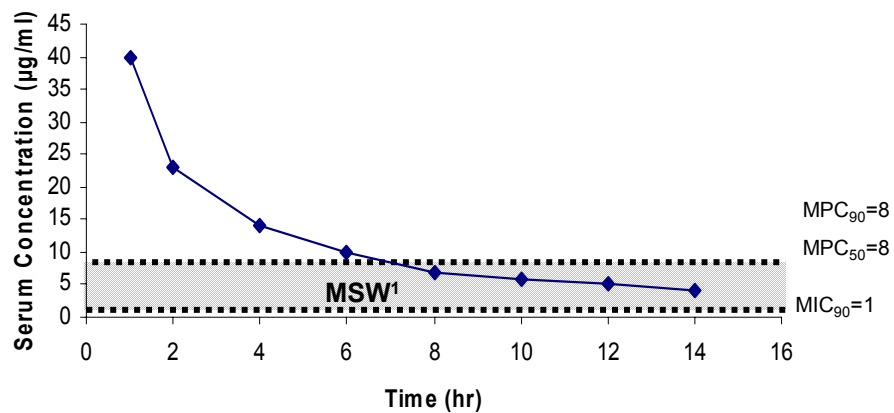
Figure 3.19: Relationship of serum concentration of moxifloxacin to the mutant-prevention concentration (µg/ml) for methicillin-resistant *S. aureus*.



Healy *et al*, 1987

¹MSW=Mutant-Selection Window

Figure 3.20: Relationship of serum concentration of vancomycin to the mutant-prevention concentration (µg/ml) for methicillin-susceptible *S. aureus*.



Healy *et al*, 1987

¹MSW=Mutant-Selection Window

Figure 3.21: Relationship of serum concentration of vancomycin to the mutant-prevention concentration (µg/ml) for methicillin-resistant *S. aureus*.

3.7.1.3 β -lactams

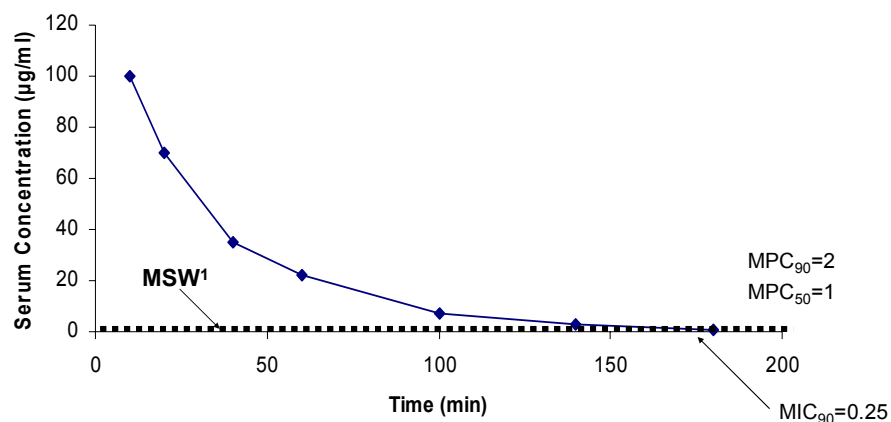
3.7.1.3.1 Cloxacillin

For cloxacillin against MSSA, the MSW was 0.25-2 $\mu\text{g/ml}$ and involved only a small portion of the pharmacokinetic curve at an i.v. dose of 500 mg over 10 min (Figure 3.22) (Soldin *et al*, 1980). This observation supports previous literature indicating that cloxacillin is an appropriate agent for effectively eradicating a systemic MSSA infection (Turnidge and Grayson, 1993; de Gorgolas *et al*, 1995; Ribera *et al*, 1996; Somekh *et al*, 1999).

For MRSA infections, the MSW involved much of the pharmacokinetic curve and extended beyond the maximum serum concentration (Figure 3.23). The MSW was 32->512 $\mu\text{g/ml}$ while the maximum serum concentration exists at ~ 100 $\mu\text{g/ml}$. To succeed in eradication of a large MRSA population with cloxacillin, one must treat at the MPC level which is beyond the maximum serum concentration and as such, unachievable. This confirms the well-known fact that cloxacillin is inappropriate for the treatment of MRSA since methicillin-resistance also confers resistance to cloxacillin (Francioli *et al*, 1991).

3.7.1.3.2 Cefazolin

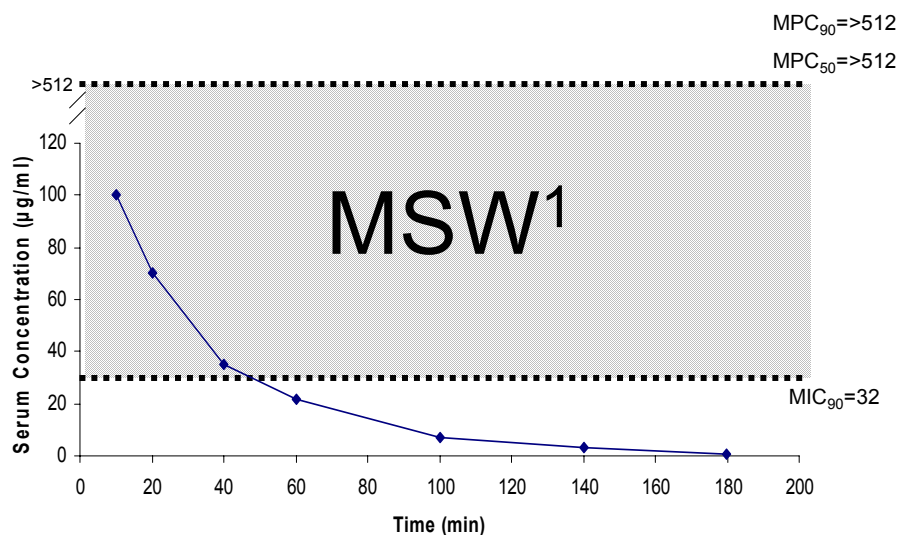
The MSW for cefazolin against MSSA and MRSA involved most of the pharmacokinetic curve at a dosage of 500 mg iv injection (Figure 3.24 and Figure 3.25) (Kirby and Regamey, 1973). For MSSA infections, the MSW was 2-64 $\mu\text{g/ml}$ and for MRSA infections, the MSW was 16-512 $\mu\text{g/ml}$. At the current recommended approved dosing, serum drug concentrations exist within the MSW for MSSA, increasing the risk of this agent selecting for resistant subpopulations, if present. Since MRSA are already resistant to cefazolin, this agent would be inappropriate for the treatment of MRSA infections, however, it is still interesting to study the MPCs in relation to the pharmacokinetics. Serum concentrations remain in the MSW for ~ 4 hr. It is possible that different levels of resistance are present within a population and that with high inocula and antimicrobial selective pressure, these higher level resistant cells may be selected from the population. The relationship of this, however, to what occurs clinically is unknown. For MSSA, the MPC₉₀ value (64 $\mu\text{g/ml}$) may be achievable since it exists below the maximum serum concentration. For MRSA, the MPC₉₀ value



Soldin *et al*, 1980

¹MSW=Mutant-Selection Window

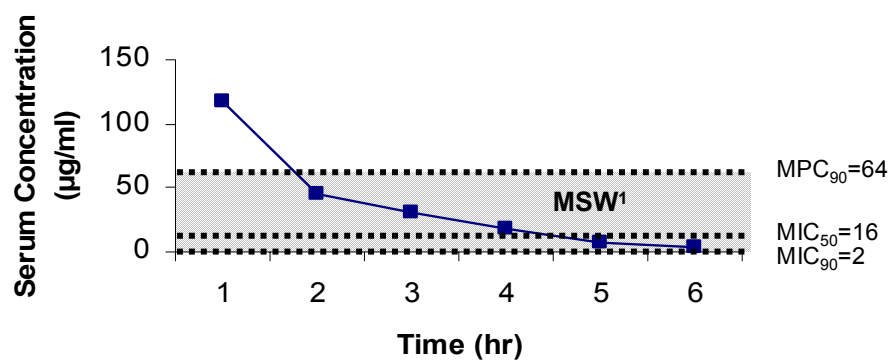
Figure 3.22: Relationship of serum concentration of cloxacillin to the mutant-prevention concentration (µg/ml) for methicillin-susceptible *S. aureus*.



Soldin *et al*, 1980

¹MSW=Mutant-Selection Window

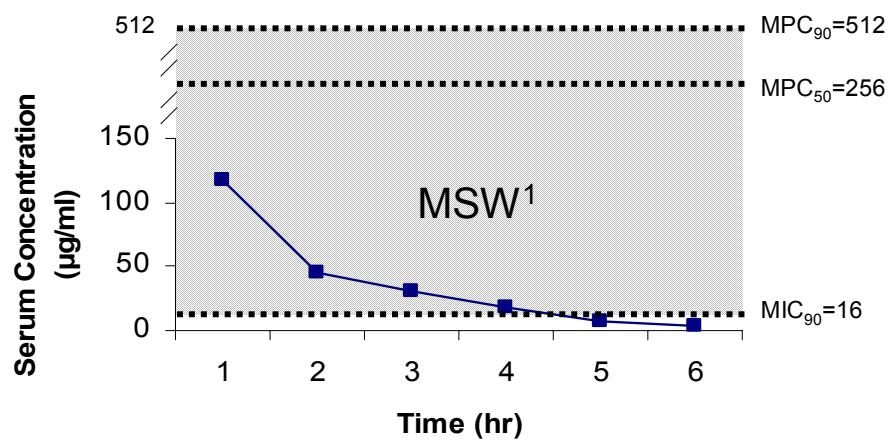
Figure 3.23: Relationship of serum concentration of cloxacillin to the mutant-prevention concentration (µg/ml) for methicillin-resistant *S. aureus*.



Kirby and Regamey, 1973

¹MSW=Mutant-Selection Window

Figure 3.24: Relationship of serum concentration of cefazolin to the mutant-prevention concentration (µg/ml) for methicillin-susceptible *S. aureus*.



Kirby and Regamey, 1973

¹MSW=Mutant-Selection Window

Figure 3.25: Relationship of serum concentration of cefazolin to the mutant-prevention concentration (µg/ml) for methicillin-resistant *S. aureus*.

(512 µg/ml) is not likely achievable, since this value is beyond the maximum serum concentration (~120 µg/ml). According to the presented *in vitro* data, cefazolin may potentially select for resistant subpopulations in MSSA infections and higher levels of resistance in MRSA infections.

3.7.1.4 Macrolide

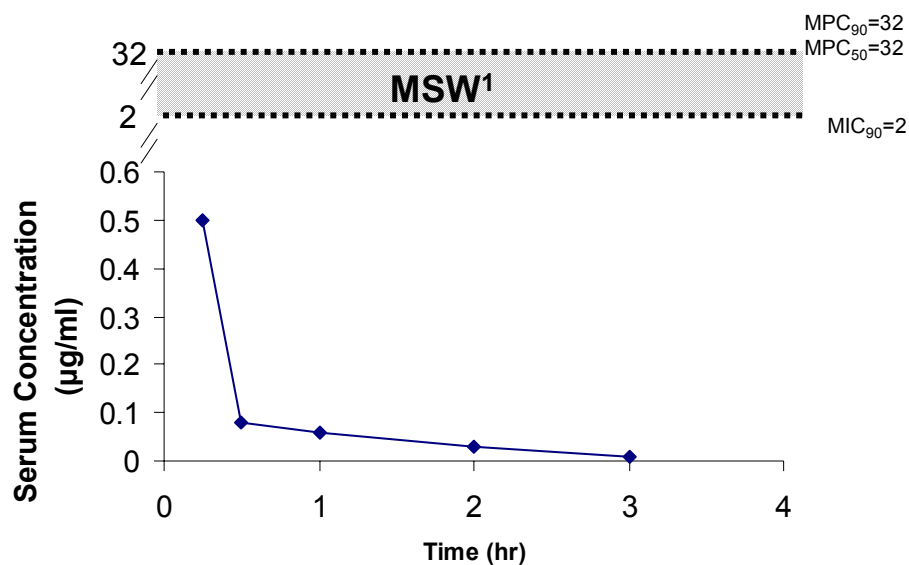
3.7.1.4.1 Azithromycin

For azithromycin, both MSSA and MRSA portrayed a MSW that extended above the maximum serum concentration (Figures 3.26 and 3.27). The MSW for both MSSA and MRSA was 2-32 µg/ml. Because the MPC₉₀ value (32 µg/ml) lies above the maximum serum concentration (0.5 µg/ml), it is unlikely that this concentration would be safely achieved in the blood. Treatment failure with azithromycin may be due to the proliferation of susceptible cells, since the serum drug concentration lies below the MIC₉₀ value for the majority of the dosing period (Tillotson *et al*, 2001).

3.8 Retrospective Multiple Sequential *S. aureus* Study

An initially susceptible population may develop into a resistant population as a result of *in vivo* selective pressure of an antimicrobial agent. As discussed earlier (section 1.4.1), resistant organisms can emerge from spontaneous mutations. In many cases, the number of bacterial organisms exceeds the spontaneous mutation frequency (Gould and MacKenzie, 2002), giving rise to resistant organisms that may selectively be enriched by an antimicrobial agent. This process has been shown to exist in the laboratory setting (Firsov *et al*, 2003). Few studies have been performed on the susceptibility behavior of multiple organisms isolated from patients who have failed antimicrobial therapy. This information may help provide evidence of a bacterial population changing its susceptibility patterns within a patient while the patient undergoes antimicrobial therapy.

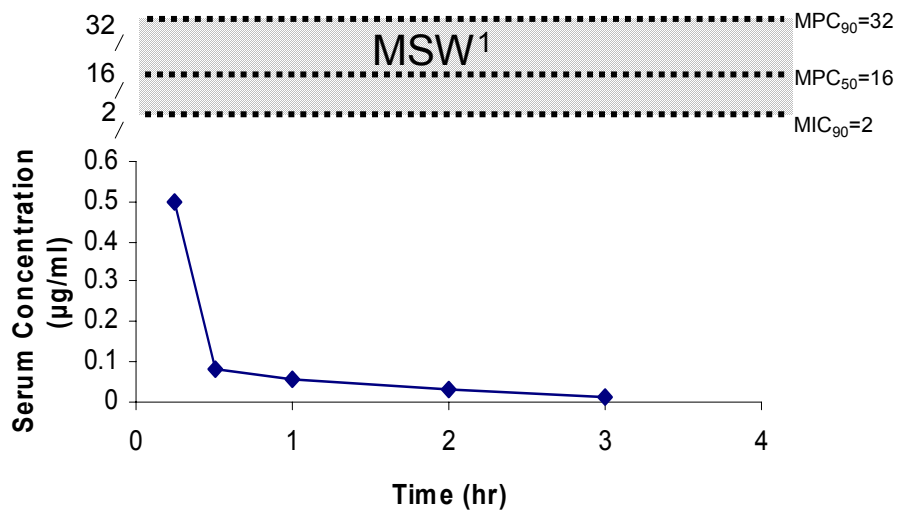
Twenty-eight multiple sequential isolates were collected from nine different patients. Isolates were collected from November (2001) to February (2002) for patients #1 and #6. Patient #2 had isolates that were collected between October (2001) and January (2002). Isolates from patients #3 and #4 were collected between the months of December (2001) and January (2002). For patients #5 and #8 isolates were collected in the months of November (2001) and December (2001), respectively. Isolates from



Pfizer Canada Inc.

¹MSW=Mutant-Selection Window

Figure 3.26: Relationship of serum concentration of azithromycin to the mutant-prevention concentration (µg/ml) for methicillin-susceptible *S. aureus*.



Pfizer Canada Inc.

¹MSW=Mutant-Selection Window

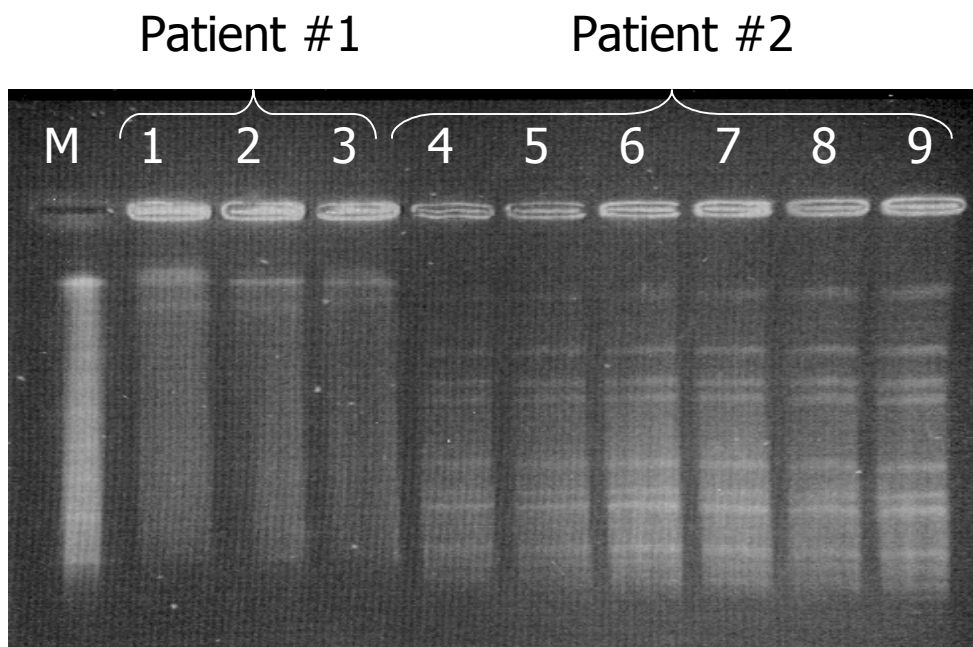
Figure 3.27: Relationship of serum concentration of azithromycin to the mutant-prevention concentration (µg/ml) for methicillin-resistant *S. aureus*.

patient #7 were collected between February (2002) and March (2002) and isolates from patient #9 were collected between December (2001) and February (2002). MIC and MPC values were determined for each isolate against the antimicrobials prescribed to the patients as well as other agents of interest.

PFGE was performed on each isolate to ensure the sequential infecting isolates were from the same origin (same strain of *S. aureus*). Figures 3.28 and 3.29 display the PFGE results. Patients one, two, three, five, six, seven, eight, and nine showed similar band patterns amongst the sequential isolates, suggesting that the infecting organisms were, indeed, the same strain of *S. aureus* throughout the duration of infection and treatment. In patient four, isolates #12 and #16 had unique band patterns when compared to the other organisms isolated from this patient, suggesting that these two strains of *S. aureus* were different than the other strains (isolates #13-15). Patient four was likely infected with more than one strain of *S. aureus*. The other patients, however, appeared to be infected with the same strain of *S. aureus* throughout the duration of their infection. With the knowledge of the same infecting strain, any differences observed in MPC values may be due to this population of cells developing resistance through selective therapy.

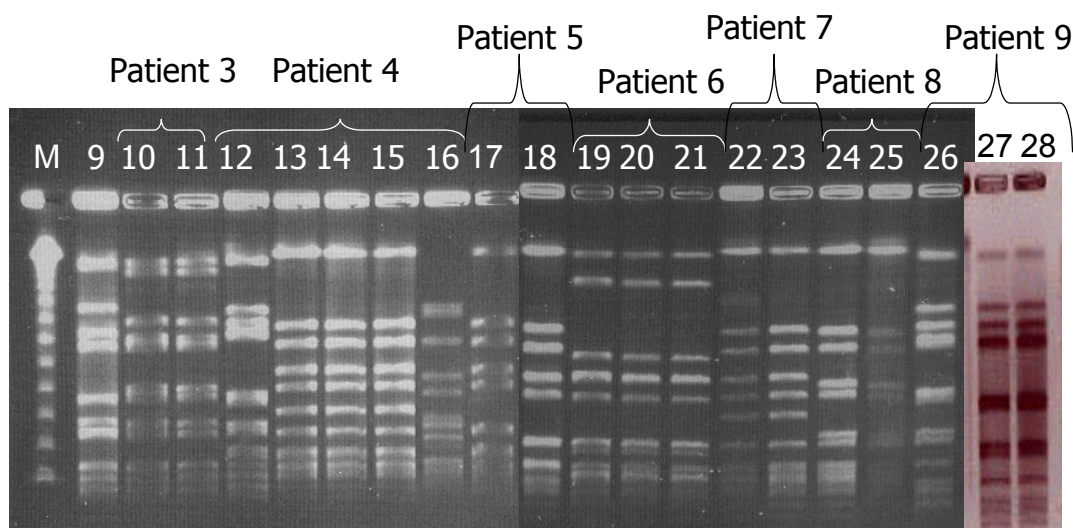
Information regarding patient antimicrobial therapy and the ability of isolates to produce β -lactamase was assessed. The agents prescribed to each patient are shown in Table 3.10. The isolates were tested for their ability to produce the enzyme, β -lactamase using the Cefinase test. The data are shown in Table 3.11. In patient four, isolate #12 was negative for β -lactamase production while isolates #13-16 were positive. This may be explained by the PFGE results which indicated that isolate #12 was a different strain compared to the other isolates collected from this patient.

Table 3.12 displays the MPC data of the retrospective sequential isolates. For clindamycin and rifampin, there appeared to be no MPC endpoint and even if so, it would likely be beyond clinically achievable drug concentrations. This phenomenon was discussed previously with rifampin (Allen, 2003). Seven small increases in MPC among sequential isolates were observed against the drugs prescribed to the patients. The last two *S. aureus* organisms isolated in patient one have 2-fold higher MPC values to cefotaxime than the original bacterial isolate (4 $\mu\text{g/ml}$ vs. 2 $\mu\text{g/ml}$). This patient was



M=Marker

Figure 3.28 Pulsed field gel electrophoresis (PFGE) on sequential multiple isolates of *S. aureus* from patients 1 and 2.



M=Marker

Lane 9 = Repeat of isolate #9 from patient 2 (refer to figure 3.28)

Figure 3.29: Pulsed field gel electrophoresis (PFGE) on sequential multiple isolates of *S. aureus* from patients 3-9.

Table 3.10: Antimicrobial history of patients from whom sequential *S. aureus* isolates were recovered.

Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
Pip/Tazo ^a	Rifampin	Gentamicin	Cloxacillin	Cefuroxime	Pip/Tazo ^a	Not Available	Cefazolin	Cephalexin
Clindamycin				Cefotaxime	Vancomycin		Gentamicin	Ciprofloxacin
Ciprofloxacin				Clindamycin	Cefazolin			Cloxacillin
Cefazolin				Cloxacillin				
Cefotaxime				Gentamicin				

^aPip/Tazo=Piperacillin/Tazobactam

Table 3.11: Detection of β -lactamase enzyme from *S. aureus* isolates using the cefinase test.

Isolate #	Result	
1	Negative	Patient 1
2	Negative	
3	Negative	
4	Negative	Patient 2
5	Negative	
6	Negative	
7	Negative	
8	Negative	
9	Negative	Patient 3
10	Positive	
11	Positive	Patient 4
12	Negative	
13	Positive	
14	Positive	
15	Positive	
16	Positive	Patient 5
17	Positive	
18	Positive	Patient 6
19	Positive	
20	Positive	Patient 7
21	Positive	
22	Positive	Patient 8
23	Positive	
24	Positive	Patient 9
25	Positive	
26	Negative	
27	Negative	
28	Negative	
29213 ^a	Positive	
10211 ^b	Negative	

^a29213=Positive Control

^b10211=Negative Control

Table 3.12: Mutant-prevention concentration (MPC) data on the sequential *S. aureus* isolates (µg/ml).

	<i>Isolate</i>	<i>Cefazolin</i>	<i>Cefotaxime</i>	<i>Cefuroxime</i>	<i>Cephalexin</i>	<i>Cipro.</i> ¹	<i>Clind.</i> ²	<i>Cloxacillin</i>	<i>Gentamicin</i>	<i>Rifampin</i>	<i>Pip/Tazo</i> ³	<i>Vanco.</i> ⁴
Patient 1	1	1	2	2	16	16	>2	0.5	>512	>16	1	4
	2	1	4	2	16	16	>2	0.5	>512	>16	2	4
	3	1	4	2	32	16	>2	0.5	32	>16	1	4
Patient 2	4	1	4	2	16	4	>2	1	>512	>16	1	4
	5	1	8	2	16	2	>2	0.5	>512	>16	1	4
	6	1	4	2	16	2	>2	0.5	>512	>16	1	4
	7	0.5	4	2	16	2	>2	0.5	>512	>16	2	4
	8	0.5	4	2	16	2	>2	0.5	>512	>16	2	4
	9	0.5	4	2	16	2	>2	0.5	>512	>16	1	4
	10	128	4	4	128	2	>2	1	8	>16	32	4
Patient 3	11	128	4	4	128	2	>2	2	8	>16	32	4
	12	4	8	4	128	2	>2	1	16	>16	32	4
	13	1	2	2	16	4	>2	0.25	64	>16	2	4
	14	1	2	1	16	4	>2	0.5	64	>16	4	4
	15	1	2	1	16	4	>2	0.5	64	>16	2	8
Patient 4	16	128	>32	>32	128	64	>2	>4	16	>16	128	4
	17	4	2	2	64	1	>2	1	8	>16	16	4
	18	64	2	2	128	2	>2	1	8	>16	16	4
Patient 5	19	32	4	2	32	2	>2	0.5	>512	>16	8	4
	20	32	4	2	64	2	>2	0.5	>512	>16	16	4
	21	16	4	2	64	2	>2	0.5	>512	>16	16	8
Patient 6	22	64	2	2	128	4	>2	1	16	>16	16	8
	23	32	4	2	64	4	>2	1	16	>16	16	4
Patient 7	24	1	2	1	32	4	>2	0.5	16	>16	16	8
	25	1	2	1	16	2	>2	0.5	16	>16	16	8
Patient 8	26	2	8	4	64	2	>2	1	16	>16	64	4
	27	2	8	4	128	2	>2	1	16	>16	64	4
	28	2	8	4	128	4	>2	1	16	>16	64	16
Control	29213	2	4	2	32	2	>2	0.5	16	>16	4	16

¹Cipro.=Ciprofloxacin

²Clind.=Clindamycin

³Pip/Tazo=Piperacillin/Tazobactam

⁴Vanco.=Vancomycin

also treated with piperacillin/tazobactam and a 2-fold increase was observed in the second isolate to this drug (2 µg/ml vs. 1 µg/ml). Patient six was treated with piperacillin/tazobactam and showed a 2-fold increase in the second and third organisms isolated when compared to the first isolate collected (16 µg/ml vs. 8 µg/ml). Vancomycin was also used in patient six and a 2-fold increase was observed in the third organism isolated (8 µg/ml vs. 4 µg/ml). The second isolate from patient seven showed a 2-fold increase in MPC with cefotaxime compared to the first isolate (4 µg/ml vs. 2 µg/ml). Patient nine was treated with both cephalexin and ciprofloxacin and increases in MPC were observed with both of these agents (cephalexin: >64 µg/ml vs. 64 µg/ml and ciprofloxacin: 4 µg/ml vs. 2 µg/ml). Interesting MPC data were observed from patient four, since the first and last organism isolated from this patient (#12 and #16, respectively) showed a reduced susceptibility by MPC with many of the antimicrobials compared to the other organisms isolated from this patient (#13-#15). The PFGE pattern of these isolates clearly showed that isolate #12 and #16 were a different species than #13-15. The different speciation most likely accounts for the different MPC values.

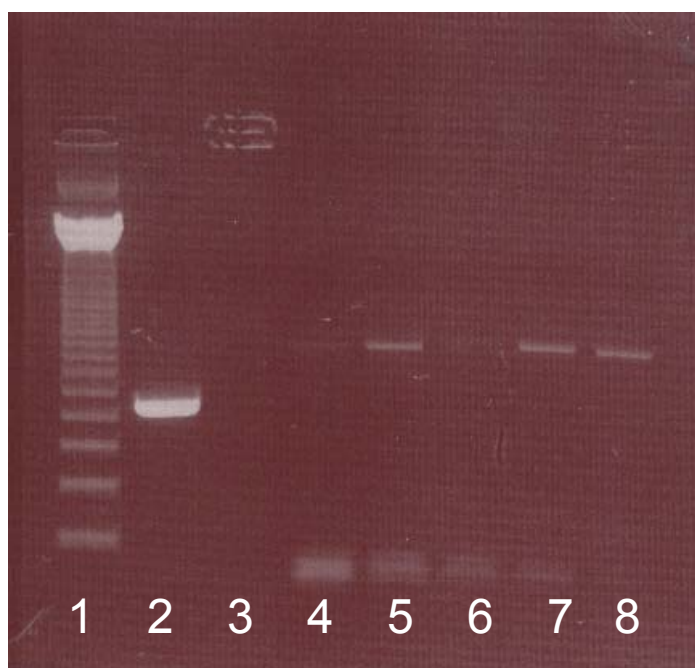
It is possible that the small increases in MPC values observed in the patients may only be due to chance or method reproducibility of the MPC test. The reproducibility of the MPC test is consistent with the reproducibility of the MIC test, that is, one doubling dilution above and below the observed value (NCCLS, 2001c,d). However, further characterization of the isolates that resulted in elevated MPC organisms was necessary to verify if, indeed, the observed elevations were due to the reproducibility of MPC test.

3.8.1 Isolate Characterization

The *mecA* gene codes for a PBP which has a low affinity for β-lactam agents. This mechanism of resistance is the most common for organisms that are resistant to β-lactam antimicrobial agents. Cefotaxime, cephalexin, and piperacillin/tazobactam are β-lactam antibiotics. Patients one, six, seven, and nine had organism isolates that were collected later during infection which showed 2-fold increases in MPC compared with the organisms collected early on in infection. These patients were treated with the β-lactam agents mentioned above. The isolates that had a 2-fold increase in MPC were

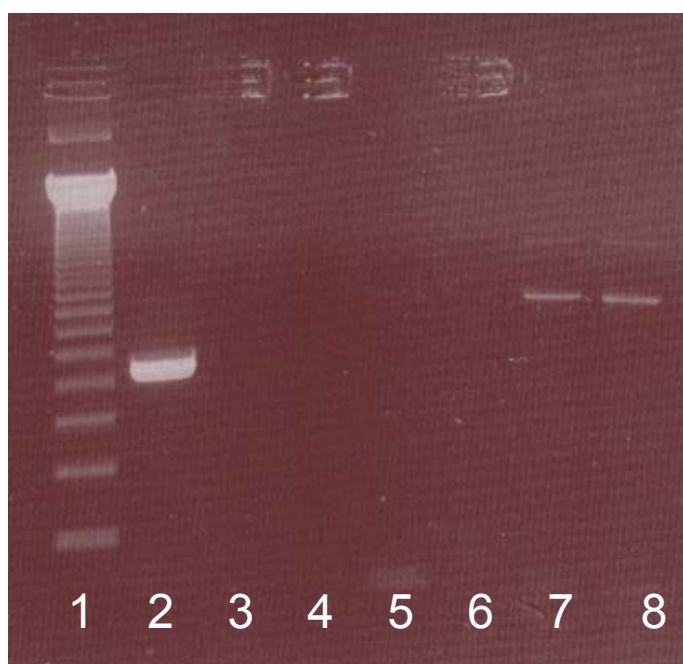
tested for the presence of the *mecA* gene. The *mecA* positive control was provided by Dr. Harry Deneer, Head of Molecular Diagnostic Pathology at RUH and the University of Saskatchewan. Each sample tested was negative for the *mecA* gene (Figures 3.30 and 3.31). Results from the isolate characterization were discouraging and, therefore, the 2-fold increases observed in vancomycin and ciprofloxacin remain unexplained. The lack of a large MPC increase as well as the lack of *mecA* in the organisms with elevated MPC values to β -lactams, led to the notion that the increases observed in vancomycin and ciprofloxacin were most likely due to the reproducibility of the MPC test (Blondeau *et al*, 2001), however, further investigations are still necessary.

This study was based on a small sample size (9 patients). To completely assess the hypothesis that resistant subpopulations may be selected from large, heterogeneous populations, *in vivo*, a larger sample size is needed. Various other confounding factors may play a role in the behavior of an infecting organism during the course of infection and increasing the sample size and stratifying the variables may help to make the experiment more valid and reliable.



Lane 1=123 bp Marker
 Lane 2=*mecA*-Positive Control
 Lane 3=29213 Negative Control
 Lane 4=MPC-elevated #1
 Lane 5=MPC-elevated #2
 Lane 6=MPC-elevated #3
 Lane 7=MPC-elevated #22
 Lane 8=MPC-elevated #23

Figure 3.30: Polymerase chain reaction (PCR) for *mecA* in sequential *S. aureus* isolates with elevated MPC values to cefotaxime (MPC-elevated).



Lane 1=123 bp Marker
 Lane 2=*mecA*-Positive Control
 Lane 3=29213 Negative Control
 Lane 4=MPC-elevated #27 (from Cephalexin MPC plate)
 Lane 5=MPC-elevated #28 (from Cephalexin MPC plate)
 Lane 6=MPC-elevated #2 (from Piperacillin/Tazobactam MPC plate)
 Lane 7=MPC-elevated #20 (from Piperacillin/Tazobactam MPC plate)
 Lane 8=MPC-elevated #21 (from Piperacillin/Tazobactam MPC plate)

Figure 3.31: Polymerase chain reaction (PCR) for *mecA* in sequential *S. aureus* isolates with elevated MPC values to cephalexin and piperacillin/tazobactam (MPC-elevated).

4.0 DISCUSSION

S. aureus is a ubiquitous organism causing worldwide morbidity and mortality (Murray *et al*, 1998c). Its ability to rapidly develop resistance has compromised the use of many antimicrobial agents. Literature suggests that the inappropriate use of an agent leads to increased resistance (Drlica and Schmitz, 2002). With increasing resistance worldwide, it is important for healthcare professionals to maintain the efficacy of antimicrobial agents through proper usage of agents (Drlica and Schmitz, 2002).

Dosing strategies are currently based on several variables, including MIC susceptibility testing, and achievable and sustainable drug concentrations. Advantages of standardized MIC testing have resulted in its worldwide acceptability (Gould and MacKenzie, 2002). Many isolates may be tested at one time making this procedure efficient. Also, with MIC testing, results are available within 24 hr. Rapid data collection is important for patients with serious bacterial infections. Another advantage of MIC testing is that it is standardized and requires few laboratory materials, thereby reducing laboratory costs. Also, microbroth dilution, E-tests, and Kirby-Bauer Disc Tests are MIC procedures that are relatively simplistic. With its efficiency, reproducibility, rapidness, simplicity, and minimal cost, MIC testing quickly became the acceptable procedure for susceptibility testing.

MIC testing, nonetheless, has some disadvantages. The procedure tests organisms at an inoculum of $\sim 10^5$ cfu/ml. Often, in an infected patient, inocula as high as 10^{10} cfu/ml exist (Gould and MacKenzie, 2002). With the spontaneous mutation frequency at one mutation for every 10^6 - 10^8 cells, inocula exceeding 10^6 cells have the potential to possess bacterial cells that have developed resistance via point mutations (Zhao and Drlica, 2001). Testing with an inoculum of 10^5 cfu/ml may not be representative of the true susceptibility of the population when $>10^5$ cfu/ml exist at the site of infection (Blondeau, 2003). It is known that large populations often contain resistant subpopulations (Allen, 2003). The MIC may not predict the development of resistance, since the MIC utilizes a small inoculum in which resistant subpopulations

are most likely absent. The MIC, therefore, may not accurately represent a drug concentration that will destroy all the cells in the population of infecting bacteria. Because resistance is increasing, it is important to assess the way in which agents are administered and ensure that the procedure is optimal. New methods to help develop or modify dosing strategies are needed in order to minimize increasing resistance.

The MPC approach is novel in that it utilizes bacterial populations exceeding 10^9 cfu/ml (Zhao and Drlica, 2002; Lu *et al*, 2003; Zhao *et al*, 2003). One advantage the MPC approach has over the MIC method is that the MPC defines the drug concentration necessary to eradicate all cells, including any spontaneously arising resistant mutants (frequency of 10^{-6} - 10^{-8}) which will be detected by using such high inocula. The MPC test also provides a measurement of the drug concentration necessary to eradicate large bacterial populations. The MPC is a concentration that could potentially prevent growth of all susceptible and first-step resistant phenotypes in a bacterial population. Dosing strategies based on MPC testing may essentially slow down emerging resistance.

There are some disadvantages to MPC testing. The procedure is lengthy and inefficient and is, therefore, not easily performed by most laboratories currently performing susceptibility testing. Few isolates can be tested in one experiment. The entire MPC test requires approximately four days as opposed to the MIC test which requires only 24 hr. The procedure also requires the use of many materials which increases laboratory costs. As such, a less demanding protocol for the MPC will be necessary before implementing the MPC test in laboratories. This is currently under investigation.

Few MPC studies have been performed on agents other than the quinolones and few studies have determined MPC values against *S. aureus* (Metzler *et al*, in press; Zhao and Drlica, 2001). It was, therefore, important to assess the applicability of the MPC to the organism, *S. aureus*, against both quinolone and non-quinolone agents. With the help of the MSW concept, MPC values may aid one in determining more potent agents as well as the agents least likely to select for resistance (Zhao and Drlica, 2002). Also, the MPC approach may help develop new dosing strategies that are aimed towards resistance prevention.

Interest was initially in determining MPC values of methicillin-susceptible and methicillin-resistant strains of *S. aureus* against four fluoroquinolones. The newer fluoroquinolones (gatifloxacin, gemifloxacin, moxifloxacin) have been reported to have enhanced activity against Gram-positive pathogens than do other agents (ciprofloxacin, levofloxacin) (Blondeau, 1999; Blondeau *et al*, 2003a). Numerous investigations (summarized in Blondeau, 2001, 2002) have confirmed these observations reporting MIC₉₀ values of 0.063-0.5 µg/ml for the newer agents and 0.25-2 µg/ml for the older compounds against methicillin-susceptible strains. Determining the susceptibility patterns of MSSA and MRSA strains against non-quinolone agents became of interest during investigations with the quinolone agents.

According to the MIC and MPC data presented, the most potent agent against MSSA isolates was gemifloxacin (MIC₉₀=0.016 µg/ml and MPC₉₀=0.125 µg/ml) and the most potent agent against MRSA isolates was vancomycin (MIC₉₀=1 µg/ml and MPC₉₀=8 µg/ml). Garenoxacin also showed increased potency compared to the other agents tested in this study with an MPC₉₀ value that was equal to 8 µg/ml for MRSA isolates. With respect to the potency of the agents, the MPC values gave results similar to those derived from the MIC data. The MPC values, however, were consistently higher than the MIC values. This was one trend observed between the MIC and the MPC data. This indicates that with increased inoculum, concentrations required to completely eradicate higher density cultures are higher than those concentrations obtained from standard susceptibility testing (MIC). The increase in resistance may be due to the selection of subpopulations with higher levels of resistance compared to the majority of the population. This may be a result of the inoculum exceeding the spontaneous mutation frequency (one mutation per 10⁶-10⁸ cells) which may lead to an increase in an organism's level of resistance via point mutations (Zhao and Drlica, 2001). Naturally, the MPC values were higher than the MIC values, since a higher concentration would be needed to eliminate a more resistant population. This increase was observed for all agents including the non-quinolones. This increase was also observed in the isolates that were initially determined resistant by MIC testing (the majority being MRSA isolates). One may expect the already resistant populations to result in the same MPC value as the MIC value. This is because the MPC is defined as

the drug concentration required to eliminate first-step resistant cells present in a population (same as the MIC value of the resistant population). However, this did not occur. The original population was initially resistant (perhaps by an earlier mutation) and when the population was grown up to approximately 10^9 cfu/ml, there was a possibility that a spontaneous mutation may have occurred which further increased the level of resistance of the organism. Therefore, the concentration required to prevent growth of the population was higher than the MIC. This indicates that perhaps resistance occurred in a step-wise fashion, increasing the level of resistance upon selective pressure and an inoculum of approximately 10^9 cfu/ml. The MPC, however, is more appropriate for bacterial populations that are deemed susceptible by traditional susceptibility testing, since it is, essentially, a method aimed at the prevention of first-step resistant mutants (Blondeau, 2003). The observation that MPC values were consistently higher than MIC values, including situations where the MIC value was already resistant according to the NCCLS, suggests that large bacterial populations increase their general level of resistance when exposed to elevated levels of drug. Consequently, resistance is not an all or none phenomenon, but appears to be more reflective of a step-wise process in which an organism can gradually decrease its susceptibility to an agent.

Another general trend observed was that the MPC values for MRSA were higher than the MPC values for MSSA excluding azithromycin and vancomycin. This may be explained by multi-resistance, indicating that resistance to one agent (methicillin in the case of MRSA) may also play a role in the reduced susceptibility to other families of agents, for example, the penicillins, fluoroquinolones, and cephalosporins (Samaha-Kfoury and Araj, 2003). This phenomenon has been observed in many bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *S. pneumoniae* isolates (Blondeau, 2003; Samaha-Kfoury and Araj, 2003). The lack of elevated MRSA MPC values compared to MSSA MPC values in azithromycin and vancomycin implies that an organism's susceptibility to methicillin has no effect on the susceptibility of that organism to agents such as azithromycin and vancomycin. However, previous literature indicates that MRSA isolates are more likely to develop reduced susceptibility to vancomycin compared to

MSSA isolates (Hiramatsu, 1998; Hiramatsu *et al*, 2002; Srinivasan *et al*, 2002). On the contrary, the data observed in this study do not indicate that MRSA isolates are more likely to develop a reduced susceptibility to vancomycin compared to MSSA isolates. The elevated MRSA values to cefazolin indicate cross-resistance of *S. aureus* organisms among methicillin and cephalosporin agents. The elevated MRSA values to quinolones indicate multi-resistance of *S. aureus* organisms among methicillin and quinolone agents. According to the data presented, there was no correlation between resistance of *S. aureus* to β -lactams, such as methicillin (MRSA), and resistance to macrolides or glycopeptides. There may, however, be a correlation between *S. aureus* resistance to β -lactams and other families of antimicrobial agents, for example, the fluoroquinolones.

It has been argued that time spent above the MPC may be important for restricting the development of resistance (Blondeau *et al*, 2002b, in press; Hansen *et al*, 2003). This may appear contradictory to the well-known characteristic of fluoroquinolones, that is, their concentration-dependent antibacterial effects. Blondeau *et al* (in press) argue that maintaining drug concentrations above the MPC for as long as possible does not contradict concentration-dependent killing, but complements it. To my knowledge, kill studies with *S. aureus* (based on the MPC concept) have not yet been performed, however, such studies have been published with *S. pneumoniae* data (Blondeau *et al*, 2002b). Studies with *S. pneumoniae* suggest that maintaining drug concentration above the MPC for extended durations was necessary to cause a high reduction (kill) in viable cells. Specifically, Blondeau *et al* (2002b) suggested that drug concentrations need to be in excess of the MPC values for >4 hr to result in a >90% reduction in viable cells. Blondeau *et al* (in press) indicated that not only was concentration an important predictor of outcome, but also time spent above the MPC. The authors state that "...drugs whose concentrations remain in excess of the MSW for longer periods of time over the dosing interval would be less likely to select for resistance" (Blondeau *et al*, in press). Similar kill studies involving *S. aureus* and based on the MPC principles may show similar observations to those for *S. pneumoniae*. However, two limitations of these kill studies should be considered. First, drug elimination does not occur *in vitro* as it does *in vivo*. Second, the *in vitro* kill studies

lack immune responses generally observed *in vivo*. Regardless, these *in vitro* experiments do suggest some interesting observations that require *in vivo* confirmation. Of the fluoroquinolones, moxifloxacin showed the greatest amount of time spent above the MPC level ($T > 24$ hr) for 122 MSSA isolates, as did garenoxacin ($T > 24$ hr) for 26 MSSA isolates, at dosages recommended by the manufacturer.

Some MPC_{90} values for particular agents were ≥ 16 -fold above their corresponding MIC_{90} values. This was apparent for agents such as azithromycin (16-fold for MSSA and MRSA), cefazolin (32-fold for MSSA and MRSA), cloxacillin (> 16 -fold for MRSA), and gemifloxacin (32-fold for MRSA). MPC values that were considerably higher than the MIC values (such as those ≥ 16 -fold) suggests that to achieve and maintain drug concentrations in the serum at or above the MPC level, dosing would involve treating the patient at high drug concentrations (Blondeau, 2003). At such high concentrations, other factors must be considered including toxicity, increased side effects, and perhaps patient compliance if the dosing regimen requires the patient to administer the agent ≥ 3 times a day. These data, therefore, must be assessed carefully and certain measures should be taken to ensure safety before treatment at high doses.

Pharmacokinetic curves of the agents as well as MIC_{90} and MPC_{90} values provide useful information with potential clinical applications. Based on the theory that the MPC prevents the growth of first-step resistant mutants, one may use these values to help determine agents least likely to select for resistance or agents that may safely be administered at or above the MPC (Blondeau *et al*, 2001). Based on the MPC concept, the antimicrobial agent least likely to select for resistance is one which is potent and has a narrow MSW (Zhao and Drlica, 2001). The MSW includes the concentrations of agent that exist between the MIC and MPC value (Zhao and Drlica, 2001). With the help of pharmacokinetic curves, one can determine the amount of time the serum concentration is within the MSW. Time spent in the MSW is a concern, since it has been shown that concentrations within this window lead to the selection of resistant subpopulations (Firsov *et al*, 2003). Based on the data presented in this study and on the MPC/MSW concept, agents least likely to select for resistant subpopulations for bacteremic MSSA infections were moxifloxacin > vancomycin > cloxacillin >

gemifloxacin > garenoxacin > gatifloxacin = levofloxacin > cefazolin. Azithromycin is not normally used for bacteremic infections since serum concentrations do not achieve necessary levels for eradication (MIC values are often above the maximum serum concentration). For bacteremic MRSA infections, the agent least likely to select for resistance was vancomycin. The next agent least likely to select for resistance was garenoxacin since the MPC level is below the maximum serum concentration indicating treatment based on MPC levels may be clinically achievable without additional adverse effects. Garenoxacin is still an investigational compound and is yet to be approved for clinical use. The remaining agents had MPC₉₀ values above the maximum serum concentration. Out of these seven agents, the agent with the least amount of time within the MSW was cloxacillin (~50 min) followed by moxifloxacin and cefazolin (serum concentrations are within the MSW for ~3 hr and ~4 hr, respectively). The remaining six agents all had MIC₉₀ values above the maximum serum concentration. These data suggest that use of various quinolones for MRSA infections is questionable. Applying the MPC data with the pharmacokinetic curves and the concept of the MSW, the most appropriate agent to use for systemic MSSA and MRSA infections based on the data in this study were moxifloxacin and vancomycin, respectively. Clinical trials/studies, however, are necessary to confirm the *in vitro* results described in this study. At this point, it remains speculative that these *in vitro* results would have similar outcomes *in vivo*.

The MPC/MSW data analyzed were for drug concentrations within serum (systemic or bacteremic infections). Often, antimicrobial agents are absorbed from the blood into other anatomical sites, thereby increasing drug concentrations at these sites. For example, garenoxacin concentrations achievable in alveolar macrophages are much higher than the maximum serum concentration (158.6 µg/ml after 2.2 hr of a single oral dose of 600 mg versus 9 µg/ml in the serum) (Andrews *et al*, 2003). After 11.5 hr, the garenoxacin concentrations in alveolar macrophages are still relatively high at 76 µg/ml (Andrews *et al*, 2003). Data showing garenoxacin concentrations which exceed the MPC₉₀ indicate that at that site of infection, alveolar macrophages in this case, the MPC may be safely achievable and effective for both MSSA and MRSA (Andrews *et al*, 2003). Also, Honeybourne *et al* (2001) observed gatifloxacin concentrations exceeding

the MPC₉₀ for both MSSA and MRSA in alveolar macrophages. Alveolar macrophages had an average drug concentration of 69 µg/ml 2 hr after a single 400 mg oral dose. After 4 and 12 hr, drug concentrations in the alveolar macrophages were still elevated at 77 and 62 µg/ml, respectively. Like garenoxacin, gatifloxacin achieves high drug concentrations in the alveolar macrophages compared to the serum. This indicates that MPC₉₀ values may be achievable and effective when the infection is at the site of alveolar macrophages. Moxifloxacin has also shown high drug concentrations in sites other than the blood. Following a single 400 mg oral dose, after 2.2 hr, concentrations in the epithelial lining fluid, the alveolar macrophages, and bronchial mucosa are 20.7, 56.7, and 5.36 µg/ml, respectively (Soman *et al*, 1999). For MSSA, these concentrations are above the MPC₉₀ level (0.5 µg/ml) and MPC₉₀ levels may, therefore, be achievable and effective at eliminating an infection within these sites based on the MPC model. For MRSA, concentrations may be achieved above the MPC₉₀ in epithelial lining fluid and in alveolar macrophages, however, not in bronchial mucosa, since the maximum concentration was only 5.36 µg/ml and the MRSA MPC₉₀ was 16 µg/ml for moxifloxacin. For infections at anatomical sites other than the blood, maximum drug concentrations differ from serum drug concentrations for azithromycin as well (Rodvold *et al*, 2003). After a 500 mg dose once daily for five days, Rodvold *et al* (2003) recorded azithromycin concentrations in the pulmonary sites of healthy volunteers. At 4, 12, and 24 hr, azithromycin concentrations were 650±259, 669±311, and 734±770 µg/ml, respectively, in alveolar macrophages.

This information indicates that although MPC₉₀ values may be unachievable in the serum, these values may be achievable at other sites (such as the epithelial lining fluid and alveolar macrophages) based on maximum drug values at the site(s) of interest. Note the pharmacokinetic curves displayed in this study indicate antimicrobial concentrations within serum. Nonetheless, plotting of the MIC₉₀ and the MPC₉₀ as well as the MSW on these curves provides insight as to the potential effectiveness of these agents for bacteremic infections.

Overcrowding was assessed with levofloxacin, a fluoroquinolone, and *S. aureus* isolate, #4. According to the overcrowding data displayed in Table 3.7, the appropriate volumes to use for MPC testing would be between 25 and 150 µl, since these volumes

provided consistent results. Also, it was previously determined in the laboratory that for *S. aureus* isolates, 100 µl of a culture containing $\geq 10^9$ cfu/ml was appropriate for MPC testing (Metzler *et al*, in press). There was no significant difference in MPC values when plating various volumes of inoculum. The MPC values obtained, therefore, were likely the true values and not a result of overcrowding. Based on these data, overcrowding does not appear to be an issue with the MPC test using levofloxacin and perhaps other fluoroquinolones with *S. aureus*.

When subpopulations recovered from MPC plates were re-tested by MIC procedures, the results indicated a change in susceptibility of a population of cells. The MPC-recovered subpopulations displayed elevated MIC levels compared to the MIC values of the original parental population. The increase in MIC values suggests that while performing the MPC experiment, the selection of resistant subpopulations occurs. Vancomycin was the only agent that did not show this phenomenon, however, it is possible that the MPC-recovered population reverted back to a susceptible wild type population while in drug-free media (Hiramatsu *et al*, 2002). Indeed, it has been observed that VRSA tend to lose their resistant phenotype while growing in the absence of vancomycin (Hiramatsu *et al*, 2002; Cui *et al*, 2003). Before re-testing these isolates by MIC, they were grown on drug-free media. It is possible that the lack of elevated MIC values upon retesting was due to the failure to provide a constant selective media and, therefore, the MPC-recovered populations were able to revert back to the wild type, susceptible cells as described by Hiramatsu *et al* (2002) and Cui *et al* (2003). Another explanation for the lack of elevated MIC values in isolates resulting in high MPC values to vancomycin was described by Blondeau (2003) and others (Tomasz *et al*, 1970; Novak *et al*, 1999) as vancomycin tolerance. This was explained by Tomasz *et al* (1970) by the ability of bacteria to survive, but not grow in the presence of drug. Cui *et al* (2003) showed that after multiple passages of VRSA strains through drug-free media, the MIC of the strains decreased, however, the strains still had the potential to develop higher level of resistance to vancomycin (resistant subpopulations still thrived within the populations with lower MIC values). Tolerant strains may, therefore, be undetected by standard susceptibility testing (Blondeau, 2003). MPC testing, however, may be useful for the detection of these vancomycin tolerant organisms since the MPC

is designed to detect subpopulations with higher levels of resistance compared to the original population.

The results from the vancomycin study were, indeed, disconcerting. For MRSA isolates, the MPC₉₀ value fell within the range of intermediate-resistant (NCCLS, 2001a). This was disturbing since it indicated the possibility of populations of *S. aureus* to have decreased susceptibility to vancomycin. Indeed, it has been shown that high level vancomycin resistance may emerge from a *S. aureus* population initially expressing low level resistance to vancomycin (Haraga *et al*, 2002). If strains of VRSA emerge from susceptible populations, this could have an incredible impact on patients infected with MRSA strains. As discussed earlier, vancomycin is significant in treating life-threatening *S. aureus* infections, especially those with organisms resistant to β -lactam agents (Hiramatsu, 2001). Once an organism becomes resistant to vancomycin, there are limited alternatives.

Because very few studies have been performed on the MPC values of *S. aureus* against vancomycin, further characterization of the recovered mutants was necessary to determine phenotypic or genotypic differences between the original susceptible population and the MPC-recovered population. Recovered organisms at high vancomycin concentrations were evaluated by PCR and EM. Each sample was negative for all vancomycin-resistant genes tested (*vanA*, *vanB*, *vanC*, *vanC2/C3*, *vanD*, *vanE*, and *vanG*). The lack of common vancomycin-resistant genes in the MPC-recovered populations suggests that a different mechanism of resistance may be present in these isolates or that these populations were tolerant to vancomycin (Blondeau, 2003).

Statistical analysis of the cell wall thickness between the susceptible parent population and the MPC-recovered population revealed that the recovered population had a significantly thicker cell wall than the susceptible population (Mann, 1998b). This suggests that while performing the MPC test, concentrations between the MIC and the MPC values will result in the selection of a subpopulation of cells which are at a higher level of resistance towards vancomycin compared to the majority of the original population, possibly due to the thickening of the cell wall. The above data observed may indicate that *S. aureus* has the potential to become resistant to vancomycin due to dosing strategies that result in serum vancomycin concentrations within the MSW. The

clinical impact of this observation is yet to be established and further *in vitro* investigations are necessary to further explain this phenomenon.

Recovered isolates with high MPC values to azithromycin were tested for common macrolide-resistant genes (*ermA*, *ermB*, *ermC*, *mefA/E*, and *msrA/msrB*). Three of the recovered isolates tested were positive for the *ermC* gene. The parents of these isolates were negative for the *ermC* gene. A similar observation was made with *S. pneumoniae* isolates. *S. pneumoniae* MPC-recovered populations showed the presence of *mefA* or *ermB* while the parents of these isolates lacked these genes. The characterization of these isolates provided evidence that in the original susceptible population, a resistant determinant was absent or undetected, however, in the MPC-recovered population (in which a resistant subpopulation may have been selected) the resistance determinant was detectable. It is known that the mere presence of these macrolide-resistant genes confers resistance, not via a spontaneous mutation (Smith *et al*, 2003). It is, therefore, puzzling as to when, where, and how the MPC-recovered populations acquired the resistance determinants. These experiments appear to have provided valuable insight into the selection of macrolide-resistant determinants which may then be spread to other cells, thereby, conferring macrolide resistance, however, further investigation is needed.

Few studies exist on the possibility of resistant subpopulations emerging from original susceptible populations, *in vivo*. Studies of multiple sequential organisms isolated from patients at different times during the course of therapy may provide evidence of the enrichment of resistant subpopulations from large heterogeneous bacterial populations, *in vivo*. Sequential isolates from nine patients were analyzed for changes in MPC values. Small changes observed, however, were considered as insignificant when resistance determinants were absent. The MPC values of isolates have a reproducibility of one doubling dilution above and below the MPC value (Blondeau *et al*, 2001). The increases observed were, indeed, only one doubling dilution above the preceding MPC value, therefore, it is likely that the increases were due to the reproducibility of the MPC test. More studies need to be done to evaluate the possibility of resistant subpopulations emerging from large heterogeneous populations, *in vivo*.

The data presented provide a solid base for MPC data obtained with *S. aureus* organisms towards different antimicrobial agents. The MPC appears to be applicable to not only the quinolone agents, but to non-quinolones as well. For example, the data presented in this study suggest that applicability of the MPC to macrolides and to glycopeptides is a definite possibility. However, since MPC relates to mutant prevention, it may not be representative of all forms of resistance especially those that are transmissible on mobile genetic elements. For this reason, a Resistance-Prevention Concentration (RPC) may be more encompassing. Whether the RPC can impact on existing resistance or serve to prevent resistance in a fashion similar to MPC requires more extensive investigations on a broader range of antimicrobial agents and genus and species of organisms than what has been completed to date. Nonetheless, the data presented in this study provide evidence of resistance prevention at MPCs for both quinolone and non-quinolone agents as well as information regarding an agent's propensity to select for resistance in large populations of *S. aureus*.

It appears the MPC is an important parameter to consider in preventing resistance, *in vitro*. The combination of the MIC, the MPC, and the MSW are important to help assess agents least likely to select for resistance, *in vitro*. It is speculated that the MPC concept will be significant in the future when determining new dosing strategies aimed at preventing resistance in clinical cases, especially with respect to immunocompromised patients.

4.1 Future Considerations

Questions and future considerations have evolved from the data presented in this thesis. Future experimentation should be done on macrolide-resistant genes found in MPC-recovered isolates to help determine when and how these bacterial populations acquired the genes. Testing different inocula (10^5 , 10^6 , 10^7 , 10^8 , and 10^9) may help determine at which point the resistant genes are detectable.

More studies need to be performed on VRSA isolates in hopes of discovering a genetic determinant or mutation(s) for the phenomenon of the thickening of the cell wall in these VRSA isolates. A study comparing the DNA sequences of the genes involved in bacterial cell wall synthesis of VSSA and VRSA isolates may provide insight as to mechanism of resistance in VRSA isolates.

The MPC should further be assessed with non-quinolone agents to help determine its applicability to these agents. The determination of genetic differences between susceptible parent populations and resistant subpopulations would strengthen the argument that the MPC (or RPC) is applicable to non-quinolone agents.

Also, a more simplistic procedure should be developed for the MPC before its introduction into laboratories that test for antimicrobial susceptibilities. This is currently under investigation.

The MPC concept must be assessed in animal and human trials before clinical application. This can be done by determining the amount of time necessary to achieve killing of bacteria with different concentrations of drug and different bacterial loads in animal models such as rats. Recent preliminary experiments performed by Glen Hansen (personal communication) with rats indicate that resistant subpopulations can be selected out of large heterogeneous populations of cells with currently accepted dosing strategies (unpublished observation). Currently under investigation are the effects of MPC drug concentrations and $T > MPC$ in rats with bacterial loads of high magnitude ($\geq 10^8$ cfu).

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6.0 APPENDIX A

6.1 Solutions and Buffers

10X TE Buffer

Dissolve 12.1 g of Tris Base and 3.72 g of EDTA in 750 ml of distilled water. Bring to pH 8.0 using HCl. Adjust volume to 1 L and autoclave.

10X TBE

Dissolve 90.8 g of Tris Base, 15.4 g of Boric acid, and 0.37 g of disodium EDTA in 1000 ml of distilled water and autoclave.

0.5 M EDTA

Add 93.05 g of EDTA (disodium salt) to 400 ml distilled water. Add NaOH pellets one at a time until the EDTA is completely dissolved. Bring the pH to 8.0 by adding 5 M NaOH. Adjust volume to 500 ml and autoclave.

0.1M Cacodylate Buffer

Combine 16 g of sodium cacodylate (Mol. Wt. 160.0) with 920 ml of distilled water. pH to 7.2 with 0.1N HCL. Adjust to 1000 ml with distilled water.

10% Sarcosyl

Dissolve 10 g of sodium sarcosinate in 70 ml distilled water. Use of heat will aid in dissolving the sarcosyl. Adjust volume to 100 ml and autoclave.

1% Pulsed Field Agarose

Add 1.0 g pulsed field agarose to 100 ml 0.5X TBE. Boil until agarose is dissolved and cool to 50°C before pouring the gel. Allow gel to solidify for a minimum of 30 min before use.

1% Agarose Gel for PCR

Add 0.35 g to 35 ml of TBE Buffer containing ethidium bromide (ETBr). Microwave on high until the agarose is completely dissolved. Pour gel and allow it to solidify for ~30 min.

2% Glutaraldehyde

Add 10 ml of 25% Glutaraldehyde EM grade to 115 ml of 0.1M cacodylate buffer.

2% Osmium Tetroxide

Add 1 g of osmium tetroxide to 25 ml distilled water.

3% Uranyl Acetate

Mix 1.5 g of uranyl acetate with 50 ml of 30% ethanol for at least 30 min and protect from light.

Cell Suspension Buffer

Combine 100 μ l of 1M Tris-HCl (pH 7.2), 200 μ l of 1M NaCl, and 1.0 ml of 0.5M EDTA (pH 8.0). Dilute to 10 ml with sterile distilled water.

Epon-Araldite

Mix 81 g of Epon 812, 66 g of Araldite 502, and 180 g DDSA. Then add 4.5 g of DMP30 and mix well.

ETBr Buffer

Add 50 μ l of ETBr at 10 mg/ml to 1 L of TBE Buffer for a final ETBr concentration of 500 μ g/ml.

ETBr Solution For Staining a Pulsed Field Gel

Combine 40 μ l stock ethidium bromide with 400 ml of distilled of distilled water.

Low Melting Point Agarose

Dissolve 1 g into 100 ml of TE Buffer at pH 8.0. Boil for 1-1.5 min and cool to 50-65°C in water bath.

Lysis Buffer

Combine 100 μ l of 1M Tris-HCl (pH 7.2), 500 μ l of 1M NaCl, and 1.0 ml of 0.5M EDTA (pH 8.0). Weigh out 20 mg of deoxycholate and 50 mg of N-Lauroylsarcosine and add to above mixture. Dilute to 10 ml with sterile distilled water.

PFGE Running Buffer

Dilute 300 ml of 10X TBE in 2700 ml of distilled water for a concentration of 1X TBE.

Proteinase K Buffer

Combine 50 ml of 0.5M EDTA (pH 8.0) and 10 ml of 10% N-Lauroylsarcosine. Adjust pH to 9.0, then dilute to 100 ml with sterile distilled water.

Proteinase K/Proteinase K Buffer

Add 25 μ l of 20 mg/ml of Proteinase K to 10 ml of Proteinase K Buffer.

Reynold's Lead Citrate

Mix 1.33 g lead acetate, 1.76 g sodium citrate, and 30 ml of double distilled water that has been boiled to remove CO₂. Shake vigorously for 1 minute then place on a magnetic stirrer for 30 min. Add 8 ml of 1N NaOH (prepared fresh using 2.135 g of NaOH and 50 ml of double distilled deionized water). Adjust to 50 ml using boiled deionized water. Mix well.

Skim Milk

Dissolve 200 g of powdered skim milk into 1000 ml of distilled water and autoclave.

TE Buffer

Add 5 ml of 1M Tris-HCl pH 8.0 and 8 ml of 0.25M EDTA pH 8.0 into 494 ml of distilled water.

Toluidine Blue

Mix 2.5 g of Toluidine Blue, 2.5 g of sodium borate, and 250 ml of distilled water for several hr.

Tracking Dye

Add 60 g of sucrose into 100 ml sterile distilled water to make a 60% sucrose mixture. Add 0.25 g of xylene cyanol into 100 ml sterile distilled water. Add 10mM Tris at pH 8.0. Combine the 60% sucrose, the 0.25% xylene cyanol, and the Tris.

TSA

Add 40 g to 1 L distilled water. Autoclave.

Wash Buffer

Combine 10 ml of 1M Tris-HCl (pH 7.6) and 20 ml of 0.5M EDTA (pH 8.0). Dilute to 100 ml with sterile distilled water.

7.0 APPENDIX B

7.1 PFGE Conditions

Initial Time: 5.3 seconds
Final Time: 34.9 seconds
Included Angle: 120
Voltage: 200V, 6V/cm
Run Time: 18 hr
Variant Speed Pump: 55

8.0 APPENDIX C

8.1 Preparation of Antimicrobial Agents

Azithromycin

Add 0.02 g of powdered agent to 2 ml of 95% alcohol. At 94.5% purity, the final stock concentration is 9450 µg/ml.

Cefazolin

Add 2.5 ml sterile distilled water to 1000 mg of cefazolin. The final stock concentration is 334 mg/ml according to directions from the manufacturer.

Cefotaxime

Add 10 ml of sterile distilled water to a 500 mg vial of cefotaxime to yield a final stock concentration of 50 000 µg/ml.

Cefuroxime

Add 7.5 ml of sterile distilled water to a vial containing 750 mg of cefuroxime. The final stock concentration is equal to 100 mg/ml.

Cephalexin

Five milliliters of 1M NH₄OH is added to 0.05 g for a final stock concentration of 10 000 µg/ml.

Ciprofloxacin

Add 0.109 g to 9.896 ml of sterile distilled water for a final stock concentration of 10 000 µg/ml.

Clarithromycin

Dissolve 0.01 g in 1 ml of methanol. At 97.7% purity, the final stock concentration is 9770 µg/ml.

Clindamycin

This agent is available in injection form at 150 mg/ml. Dilute to desired concentration.

Cloxacillin

Ten milliliters of sterile distilled water is added to 2.5 g of cloxacillin for a final stock concentration of 250 mg/ml.

Erythromycin

Add 0.1 g of erythromycin into 1 ml of 95% alcohol for a final stock concentration of 10 000 µg/ml.

Garenoxacin

Dissolve 0.067 g of DES into 100 µl of DMSO. Add 6.7 ml of sterile distilled water for a final stock concentration of 10 000 µg/ml.

Gatifloxacin

Dissolve 0.035 g into 10 ml of sterile distilled water. At 93.3% purity, the final stock concentration is 3265.5 µg/ml.

Gemifloxacin

Eight milliliters of sterile distilled water is added to 0.1 g of powdered gatifloxacin. At 75.4% purity, the final stock concentration is 9425 µg/ml

Gentamicin

Gentamicin is available in the injection form at 40 mg/ml. Dilute to desired concentration.

Levofloxacin

This agent is available in injection form at 25 mg/ml. Dilute to desired concentration.

Moxifloxacin

Add 0.015 g into 10 ml of sterile distilled water for a final stock concentration of 1 500 µg/ml.

Rifampin

Add 0.05 g into 5 ml of DMSO. At 95% purity, the final stock concentration is 9 500 µg/ml.

Piperacillin/Tazobactam

Weigh out 0.03 g of tazocin (88.6% is piperacillin and 11.4% is tazobactam) and add to 2.64 ml of sterile distilled water. Final stock concentration of piperacillin is 10 000 µg/ml. Final stock concentration of tazobactam is 1295.5 µg/ml.

Vancomycin

Add 10 ml of sterile distilled water to a vial containing 500 mg of vancomycin for a final stock concentration of 50 000 µg/ml.

9.0 APPENDIX D

9.1 MIC Control Ranges for ATCC *S. aureus* Strain 29213

Azithromycin	0.5-2.0 µg/ml*
Cefazolin	0.25-1.0 µg/ml*
Cefotaxime	1.0-4.0 µg/ml*
Cefuroxime	0.5-2.0 µg/ml*
Cephalexin	0.12-0.5 µg/ml [†]
Ciprofloxacin	0.12-0.5 µg/ml*
Clindamycin	0.06-0.25 µg/ml*
Garenoxacin	Not Available
Gatifloxacin	0.03-0.12 µg/ml*
Gemifloxacin	0.008-0.03 µg/ml*
Gentamicin	0.12-1.0 µg/ml*
Levofloxacin	0.06-0.5 µg/ml*
Moxifloxacin	0.016-0.12 µg/ml*
Oxacillin	0.12-0.5 µg/ml*
Rifampin	0.004-0.016 µg/ml*
Piperacillin/Tazobactam	0.25/4.0-2.0/4.0 µg/ml*
Vancomycin	0.5-2.0 µg/ml*

* NCCLS, 2001d

[†] <http://www.fda.gov/cder/ogd/rld/50614s19.pdf>

9.2 MIC Control Ranges for ATCC *S. pneumoniae* Strain 49619

Azithromycin	0.06-0.25 µg/ml*
Clarithromycin	0.03-0.12 µg/ml*
Erythromycin	0.03-0.12 µg/ml*

* NCCLS, 2001c

10.0 APPENDIX E

10.1 Suppliers

10.1.1 Media

Mueller Hinton Broth (MHB)	Becton, Dickinson and Co., Sparks, MD
Todd Hewitt Broth (THB)	Becton, Dickinson and Co., Sparks, MD
Tryptic Soy Agar (TSA) with 5% Sheep Blood	Becton, Dickinson and Co., Sparks, MD

10.1.2 Antimicrobial Agents

Azithromycin	Pfizer Inc., Groton, CT
Cefazolin	Novopharm Limited, Toronto, ON
Cefotaxime	Hoeschst-Roussel Canada Inc., Montreal, QC
Cefuroxime	Eli Lilly Canada Inc., Toronto, ON
Cephalexin	Sigma Chemical, Steinheim, Germany
Clarithromycin	Abbott Chemicals, Bacceloneta, Puerto Rico
Clindamycin	SABEX, Boucherville, QC
Cloxacillin	Novopharm Limited, Toronto, ON
Clindamycin E-test strips	AB Biodisk, Solna, Switzerland
Ciprofloxacin	Bayer, West Haven, CT
Erythromycin	Novopharm Limited, Toronto, ON
Garenoxacin	Bristol Myers Squibb, Wallingford, CT
Gatifloxacin	Bristol Myers Squibb, Wallingford, CT
Gemifloxacin	GlaxoSmithKline Biologicals, Belgium
Gentamicin	Novopharm Limited, Toronto, ON
Levofloxacin	Janssen-Ortho Inc., North York, ON
Moxifloxacin	Bayer, Mt. Prospect, IL
Piperacillin/Tazobactam	Wyeth-Ayerst Canada Inc., Montreal, QC
Vancomycin	Eli Lilly Canada Inc., Toronto, ON

10.1.3 Reagents, Chemicals, and Enzymes

25% Glutaraldehyde (EM Grade)	Marivac, St. Laurent, QU
95% Alcohol	Commercial Alcohols Inc., Brampton, ON
Agarose	Invitrogen, Corisbad, CA

Ammonium Hydroxide	BDH Inc., Toronto, ON
Araldite 502	Ted Pella, Inc., Millville, NJ
Boric Acid	BDH Inc., Toronto, ON
Buffer A	New England BioLabs, Mississauga, ON
Cefinase	BBL Microbiology Systems, Cockeysville, MD
DDSA (Dodecenylsuccinic Anhydride)	Ted Pella, Inc, Millville, NJ
Deoxycholate Acid	Sigma-Aldrich Co., St. Louis, MO
DMP-30 [Tri(Dimethylaminoethyl Methacrylate)]	Ted Pella, Inc., Millville, NJ
EDTA	Sigma-Aldrich Co., St. Louis, MO
Epon 812	Electron Microscopy Sciences, Fort Washington, PA
Ethidium Bromide	BioRad Laboratories, Hercules, CA
Ethanol	Electron Microscopy Sciences, Fort Washington, PA
Hydrochloric Acid (HCl)	BDH Inc., Toronto, ON
InstaGene Matrix	BioRad Laboratories, Hercules, CA
Kodak D-19 Developer	Kodak
Kodak Rapid Fixer	Kodak
Lambda Ladder	New England BioLabs, Mississauga, ON
Lead Acetate	Anachem, Bedfordshire, UK
Lead Citrate	Ted Pella, Inc., Millville, NJ
Low Melting Point Agarose	BioRad Laboratories, Hercules, CA
Lysostaphin	Sigma Chemical Co., St. Louis, MO
Mass Ladder	Invitrogen, Burlington, ON
N-Laurylsarcosine	Sigma Chemical Co., St. Louis, MO
Osmium Tetroxide	Marivac, St. Laurent, QU
PCR Ladder	Invitrogen, Carisbad, CA
PCR Primers	Sigma-Genosys, Oakville, ON
PuReTaq Ready-To-Go PCR Beads	Amersham/Pharmacia, Piscataway, NJ
Proteinase K	Sigma Chemical Co., St. Louis, MO
Pulsed Field Certified Agarose	Sigma Chemical Co., St. Louis, MO
Saline	Baxter, Deerfield, IL
Skim Milk	Becton, Dickinson and Co., Sparks, MD
Slidex Pneumo-Kit	bioMerieux, Marcy-l'Etoile, France
<i>Sma</i> I	New England BioLabs, Mississauga, ON
Sodium Borate	Fisher Scientific, USA
Sodium Cacodylate	Ted Pella, Inc., Millville, NJ
Sodium Chloride (NaCl)	BDH Inc., Toronto, ON
Sodium Citrate	BDH Inc., Toronto, ON
Sodium Hydroxide (NaOH) Pellets	BDH Inc., Toronto, ON
Staphaurex	Remel Inc., UK

Sucrose
Tris-HCl
Uranyl Acetate
Wizard Kit
Xylene Cyanol

BDH Inc., Toronto, ON
Sigma Chemical Co., St. Louis, MO
Ted Pella, Inc., Millville, NJ
Promega, Madison, WI
BioRad Laboratories, Hercules, CA

10.1.4 Disposable Labware

200 µl Pipette Tips
Corning Cryovials
Cuvettes
Glass Tubes
Latex Gloves
McFarland Tubes
Microcentrifuge Tube
Microtitre Plates
Pasteur Pipettes
Sterile Plastic Petri Plates
Swabs
Wooden Applicator Sticks

VWR International, Edmonton, AB
Corning Inc., Corning, NY
Fisher Scientific, USA
Fisher Scientific, USA
Fisher Scientific, USA
Fisher Scientific, USA
Fisher Scientific, USA
Sarstedt, Newton, NC
Fisher Scientific, USA
Fisher Scientific, USA
Fisher Scientific, USA
Puritan, Guilford, Maine

10.1.5 Equipment

20 µl, 200 µl and 1 ml Pipettors
-70°C Freezer
Avanti J-E Centrifuge
CHEF DRIII PFGE System
Colorimeter
Hot Plate/Stirrer-Model 300T
PCR Gel Casting Mold
PFGE Gel Casting Mold
pH Meter
Pulsed Field Gel
Casting Apparatus
Gel Doc 1000 Illuminator
Microcentrifuge
Microwave Oven
Oxygen Incubator
Shaking Water Bath
Spectrophotometer
Thermocycler (PCR Express)
Vortex (Mini-Shaker Model 58)
Weigh Scale – Mettler PC440

Gilson Company, Inc., Lewis Center, OH
Forma Scientific Inc., Marjetta, OH
Beckman Coulter, Palo Alto, CA
BioRad Laboratories, Mississauga, ON
Hach Company, Loveland, CO
Fisher Scientific, USA
BioRad Laboratories, Mississauga, ON
BioRad Laboratories, Mississauga, ON
Corning Inc., Corning, NY
BioRad Laboratories, Mississauga, ON

BioRad Laboratories, Mississauga, ON
Brinkmann Instruments, Mississauga, ON
Samsung, Suwon, Korea
Hotpack Corp., Philadelphia, PA
Mandel Scientific Co., Guelph, ON
Pharmacia, Cambridge, England
Thermo Hybaid, Ashford, Middlesex, UK
Fisher Scientific, USA
DeltaRange, Zurich, Switzerland